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The SOS response of *Escherichia coli* is the prototypic DNA damage repair and cell cycle checkpoint control system. It is analogous to checkpoint control in eukaryotes. The SOS response includes blocking the cell cycle, global mutagenesis via more than one mechanism, and up regulation of DNA repair and recombination functions. Adaptive mutation is a mutational program in non-growing cells subjected to starvation and so is also a temporary mutagenic response to environmental stress. It entails global hypermutation, and previously, the signal transduction pathway from the environment to the DNA was unknown. Here we demonstrate that adaptive mutation of a *lac* allele in *E. coli* is under control of the SOS response. Although homologous recombination and recombination protein RecA (the prokaryotic homologue of hRad51 and other human DNA repair proteins) are required for *lac* adaptive mutation, we find that SOS induced levels of component(s) other than or in addition to RecA are also involved. We report a partial requirement for RecF, a recombination protein and SOS regulator, and find that the sole function of RecF in adaptive mutation is to allow SOS induction of SOS-controlled genes. We also report the discovery of an SOS-controlled factor that inhibits adaptive mutation, *psiB*. These results indicate that adaptive mutation is a tightly temporally regulated mutational response, controlled positively and negatively by the SOS system. In addition, we report preliminary results describing the role of damage-inducible DinB in adaptive mutation.

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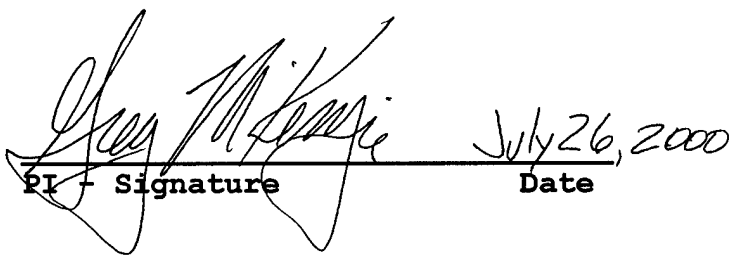

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Introduction

For the past year I have been involved in two parts of a project studying the SOS response in Stressful Lifestyle Associated Mutation (SLAM). SLAM has a number of features distinct from growth-dependent mutation including that: (i) SLAM occurs in the absence of growth while starving; (ii) SLAM occurs in a hypermutable subset of the population; (iii) SLAM has a unique sequence spectrum, being -1 deletions at mononucleotide repeats whereas growth dependent Lac^+ reversions are heterogeneous; (iv) SLAM occurs in cells in which mismatch repair is limiting; and (v) SLAM requires homologous recombination proteins RecA, RecBC and RuvABC. The assay system that we use to study SLAM uses *Escherichia coli* with a deletion of the *lac* region on the chromosome, and a *lacIΩZ* fusion carried on the F' with a +1 frameshift in it, such that these cells are phenotypically Lac^-. These cells are plated on medium containing lactose as the sole carbon source. Colonies arise over time, and are counted on each day.

Body

I have been interested in the SOS response, and how it is involved in SLAM. The SOS response is a DNA damage/cell cycle control response in *E. coli*. It works by sensing processed DNA damage (in the form of single-stranded DNA), which is coated by the protein RecA. This activates RecA, such that it acts as a co-protease to allow cleavage of several targets within the cell. These targets include the LexA repressor, the phage λ CI repressor and the UmuD translesion synthesis protein. LexA cleavage de-represses a regulon of at least 31 genes involved in DNA repair, cell division inhibition, and induced mutagenesis.

I have been following up the observation that when an uncleavable LexA mutant protein (such that the regulon can never be induced) is present within a cell, SLAM is decreased 3-5 fold. This indicates a requirement for some LexA-repressed gene for SLAM. This requirement is for a protein is other than or in addition to RecA, which is produced at constitutive levels, and is further induced by LexA cleavage. Also, the induced mutagenesis proteins UmuDC are not required at all. These were reasonable candidates, as we know that SLAM requires RecA, and UmuDC is required for UV induced mutagenesis.

I have also found that removal of the RecF protein from cells decreases SLAM to a similar level as when LexA is not cleavable. These mutations are epistatic, indicating that either RecF is involved in LexA cleavage in this system (which is known in some other systems) or that RecF is LexA repressed (which is not thought to be the case). Experiments are ongoing to distinguish these hypotheses more firmly.

Finally, I have shown that in cells lacking LexA entirely, such that LexA-repressed genes are induced constitutively, SLAM is inhibited. This indicates that in addition to genes repressed by LexA that are required for SLAM, there are gene(s) repressed by LexA that inhibit SLAM. One such gene that I have found is PsiB, a RecA co-protease inhibitor (McKenzie *et al.* 2000).

I have also been working to identify which gene or genes are involved in SLAM that are LexA repressed. An excellent candidate is the translesion synthesis DNA polymerase DinB, aka DNA polymerase IV. This gene is required for phage λ untargeted mutagenesis, and also increases the spontaneous mutation frequency over 10 fold when overexpressed. Overexpression stimulates -1 deletions at mononucleotide repeats greater than 800 fold, making it an intriguing candidate for SLAM. I have been investigating whether this gene is required for SLAM. When I knocked-out *dinB*, I found that SLAM was totally abolished. The first allele of *dinB* that I used was a polar allele that affected at least one downstream gene. I found that I was unable to complement this mutant's defect in SLAM with the *dinB* gene, indicating a requirement for other downstream genes. I have completed constructing a *dinB* point mutant that has been shown by other groups to lack any activity *in vitro* and *in vivo*. I am currently involved in examining the phenotype of this mutant, and it appears that stationary-phase mutation is decreased substantially in these cells. I expect to begin writing a manuscript on this work within a few weeks, which we will submit to Nature.

Finally, I am now interested in looking at the genes downstream of *dinB*. Homology searches show that two of the downstream genes have no homologues with defined functions. However, one gene --- *yafN* --- has homology to an anti-toxin involved in maintaining plasmids stably. This system works by producing an unstable antitoxin and a stable toxin from the same plasmid. Any cell that loses the plasmid will have the antitoxin degrade, and the toxin remain and kill the cell. YafN is homologous to several anti-toxins. As it is immediately downstream of a damage-inducible promoter, it will be interesting to see whether it is induced upon DNA damage and if it behaves as an anti-toxin as well.

I have also been involved peripherally in showing that stationary-phase mutations on the chromosome occur by a mechanism with similar requirements as that on the F' plasmid (Bull *et al.* 2000).

I have also been involved in the training of 5 rotation students, in projects that are examining the role of various helicases in recombination. I have also been mentoring a high school, now undergraduate student since June 1999. His work is part of the aforementioned PNAS paper.

Key accomplishments:

- LexA regulon involvement in SLAM (published in PNAS)
- DinB involvement in SLAM
- chromosome of *E. coli* has similar requirements for mutation as F' plasmid (published in Genetics)
- mentoring 6 students in techniques involved in studying mutation and recombination in *E. coli*

Meeting Presentations:

1999. Molecular Genetics of Bacteria and Phages Meeting, August 2-8, University of Wisconsin, Madison. The SOS response in stationary-phase mutation.

Publications:

Bull, H. J., G. J. McKenzie, P. J. Hastings and S. M. Rosenberg. 2000 Evidence That Stationary-Phase Hypermutation in the Escherichia coli Chromosome Is Promoted by Recombination. Genetics **154:** 1427-1437.

Lombardo, M-J, J Torkelson, HJ Bull, GJ McKenzie, and SM Rosenberg. 1999. Mechanisms of genome-wide hypermutation in stationary-phase. *Annals NY Acad Sci* 870: 275-289.

McKenzie, G. J., R. S. Harris, P. L. Lee and S. M. Rosenberg. 2000 The SOS response regulates adaptive mutation. Proc Natl Acad Sci U S A **97:** 6646-6651.

Conclusions

I have made substantial headway in understanding the role of the SOS response in stationary-phase adaptive mutation. I have identified the LexA-repressed inhibitors of SLAM and am in the process of identifying the LexA-repressed component that is required for SLAM. In the process I am discovering new genes that are involved in SLAM and may have interesting, novel functions in *Escherichia coli*.

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Mechanisms of Genome-Wide Hypermutation in Stationary Phase[a]

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ABSTRACT: Stationary-phase mutation (a subset of which was previously called adaptive mutation) occurs in apparently nondividing, stationary-phase cells exposed to a nonlethal genetic selection. In one experimental system, stationary-phase reversion of an *Escherichia coli* F′-borne *lac* frameshift mutation occurs by a novel molecular mechanism that requires homologous recombination functions of the RecBCD system. Chromosomal mutations at multiple loci are detected more frequently in Lac$^+$ stationary-phase revertants than in cells that were also exposed to selection but did not become Lac$^+$. Thus, mutating cells represent a subpopulation that experiences hypermutation throughout the genome. This paper summarizes current knowledge regarding stationary-phase mutation in the *lac* system. Hypotheses for the mechanism of chromosomal hypermutation are discussed, and data are presented that exclude one hypothetical mechanism in which chromosomal mutations result from Hfr formation.

STATIONARY-PHASE MUTATION IN THE LAC SYSTEM

Stationary-phase mutation occurs in apparently nondividing cells exposed to nonlethal genetic selection. In the F′ *lac* frameshift-reversion assay system in *Escherichia coli*,[6] stationary-phase mutation to Lac$^+$ can be distinguished unambiguously from normal spontaneous mutation that occurs during growth (reviewed in refs. 47 and 52). When *E. coli* carrying a +1 frameshift mutation in an F′-located *lacI-lacZ* fusion gene are plated on minimal medium with lactose as the only carbon source, Lac$^+$ revertant colonies appear 2 days after plating and continue to appear each day for at least 5–7 days. The Lac$^+$ mutants appearing from Day 3 onward carry mutations that form after exposure to the selective medium.[33] These mutations form via a novel molecular mechanism that requires homologous recombination proteins of the RecBCD system,[17,25,26] F′ transfer functions,[16,18] and is facilitated by transiently impaired postsynthesis mismatch repair.[24,31] Genetic evidence suggests that DNA polymerase III is responsible for the synthesis errors that lead to mutation.[14,23] These characteristics, as well as different mutation sequence spectra,[15,51] demonstrate that stationary-phase Lac$^+$ reversion occurs by a different molecular mechanism from reversion of the same *lac* allele during growth.

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RecBCD-DEPENDENT HOMOLOGOUS RECOMBINATION

The RecBCD enzyme is required for homologous recombination involving a linear DNA substrate, as in transductional or conjugational recombination, and also for double-strand break-repair (e.g., refs. 30 and 39). RecBCD is a potent nuclease (exonuclease V) specific for double-strand ends. It is also a helicase. *In vivo*, the nuclease activity of RecBCD is attenuated,[10,28a,38,53] and the helicase activity is thought to predominate, when RecBCD encounters a DNA sequence called Chi.[35,43,50] The helicase activity is proposed to produce single-strand DNA which is bound by the strand-exchange protein RecA and used to invade a homologous duplex DNA molecule. The RecD subunit is required for the nuclease activity of RecBC, so that nuclease activity is abolished in *recD* mutants.[1,3,8] *recD* mutants are also hyperrecombinagenic.[1,8,57] After strand-exchange intermediates are formed, the final steps of recombination involve resolution of those intermediates apparently by either the Ruv or the RecG systems.[29]

Stationary-phase Lac^+ reversion requires RecA, RecBC,[25] and the Ruv Holliday junction resolution system.[17,26] The requirement for RecBC implicates DNA double-strand breaks as a molecular intermediate in the process. Absence of RecD elevates both recombination[1,8,57] and stationary-phase mutation.[25] Absence of the RecG resolution system also elevates stationary-phase mutation, suggesting that the two resolution systems play opposing roles in mutation.[17,26] A transient block in resolution, predicted to result in elevated levels of recombination intermediates, stimulates stationary-phase reversion, implying that recombination intermediates promote mutation.[26]

An early, useful recombination-based model for the formation of the late-arising Lac^+ mutations proposed that the mutations could be formed as DNA polymerase errors that occur during DNA synthesis primed by recombination intermediates.[25] Additional evidence supporting this hypothesis is discussed below.

DNA POLYMERASE ERRORS AND INADEQUATE POSTSYNTHESIS MISMATCH REPAIR

Stationary-phase reversions of the +1 *lac* frameshift mutation have a characteristic sequence spectrum of −1 deletions in small mononucleotide repeats.[15,51] This contrasts with the heterogeneous sequences of reversions of the same frameshift allele during growth. −1 frameshifts are common DNA polymerase errors[45] that are normally repaired by the postsynthesis mismatch repair system (MMR).[37] The major replicative DNA polymerase, PolIII, is implicated in making the errors that become stationary-phase mutations.[14,23] Strains lacking mismatch repair function reproduce the stationary-phase mutation sequence spectrum during growth, implying that MMR is impaired during stationary-phase mutation.[31] Overproduction of the MMR protein MutL decreases stationary-phase mutation, but not growth-dependent mutation to Lac^+, implying that MutL function becomes limiting specifically during stationary-phase mutation.[24] This was the first evidence in any organism that MMR may not be constitutively active and suggests that modulation of MMR may be important in mutation formation in other systems. Recently, MMR activity was shown to be diminished in human cells during cell cycle arrest due to limiting nutrients.[44] How MMR activity is modulated is not yet known.

F TRANSFER FUNCTIONS AND THE ROLE OF THE F

Most Lac$^+$ stationary-phase reversion requires F transfer functions;[16,18] however, conjugation is not required.[16,42,49] Early in conjugal transfer, F-encoded proteins make a single-strand nick at the F origin of transfer (*oriT*).[62] This step could facilitate stationary-phase reversion if the single-strand nick were processed into a double-strand break to allow access by RecBCD.[28,48,49] Alternatively, some other F-encoded function(s) important for transfer might be required for stationary-phase Lac$^+$ mutation.

Early attempts to detect unselected mutations in one chromosomal gene occurring in association with Lac$^+$ stationary-phase mutation failed,[13] leading to the hypothesis that the mutational mechanism was directed specifically to the gene under selection ("directed mutation") so that only the *lac* allele on the F′ might mutate.[6,7] However, a genome-wide screen revealed unselected mutations at multiple chromosomal loci,[59] as will be described below. Thus, during conditions that promote recombination-dependent Lac$^+$ mutation, mutation is not targeted specifically to the gene under selection (see also ref. 12), or to the F′ episome. Rather, multiple genes throughout the genome experience hypermutation. Failure to detect unselected mutations previously may reflect sequence specificity or region specificity[47] of the mutational mechanism.

Recombination-dependent stationary-phase Lac$^+$ mutation was not detected at two sites in the chromosome. First, very little stationary-phase Lac$^+$ reversion is detected when the *lac* +1 frameshift allele is in the chromosome at the *E. coli lac* locus, and the reversion is *recA*-independent.[16,42] Second, a survey of the ability of various *lac* alleles to revert at one chromosomal site in *Salmonella typhimurium* revealed none that showed RecA-dependent stationary-phase reversion.[19] These results led to the hypothesis that recombination-dependent stationary-phase mutation is an F′-specific phenomenon (e.g., refs. 16, 18, and 42). However, the discovery of unselected chromosomal mutations in association with Lac$^+$ reversion on the F′[59] (see below) indicates that the chromosome and the F′ are both susceptible to mutation under selective conditions. Whether the mechanism of mutation is the same on both replicons is not yet clear, as discussed below.

GENOME-WIDE HYPERMUTATION IN STATIONARY PHASE

Stationary-phase Lac$^+$ mutations form in a hypermutable subpopulation of cells in which the entire genome is susceptible to mutation. This is inferred from experiments demonstrating that late-arising Lac$^+$ mutants have a higher frequency of mutation at multiple unselected loci than do cells that were also exposed to selection but remained Lac$^-$, or Lac$^-$ cells never exposed to selection.[59] Unselected mutations were detected at loci on the chromosome, on the F′, and on a ColE1 plasmid, indicating that multiple replicons are accessible to mutation.

All the mutations detected after selection for Lac$^+$ may be generated by a similar recombination-dependent route.[59] The frequency of associated chromosomal mutations is elevated in *recD* and *recG* mutant strains which have elevated recombination-dependent stationary-phase Lac$^+$ reversion (H. Bull, G.J. McKenzie, and S.M. Rosenberg, unpublished results). This indirect evidence suggests that recombination promotes the formation of the associated chromosomal mutations. Direct evidence is being sought as will be described. There is direct evidence for recombination-dependent stationary-phase muta-

tion of an F′-located unselected locus. A tetracycline resistance gene carrying a +1 frameshift mutation reverts to tetracycline resistance during prolonged starvation conditions identical to those used to detect Lac^+ stationary-phase mutation, and the reversion requires recombination functions and F′ transfer functions.[12] These results demonstrate that RecBC-dependent mutation occurs at a locus other than the F′-borne *lac*.

A MODEL FOR STATIONARY-PHASE MUTATION

We have proposed that the stationary-phase mutation process begins with double-strand breaks (DSBs) or double-strand ends (DSEs).[25] The source of the DSBs or DSEs could be DNA damage, stalled replication forks, single-strand nicking at *oriT*, or perhaps stationary phase or starvation-induced programmed DNA cleavage.[46,48,49] The RecBCD enzyme loads at double-strand ends and exposes single-strand DNA to RecA binding and subsequent strand invasion of homologous DNA. The source of homologous DNA could be a sister molecule or duplicated or amplified copies. Recombination intermediates with a 3′ strand invasion could prime DNA synthesis. DNA polymerase errors become mutations when mismatch repair is inadequate. This model predicts that DNA synthesis is directly associated with recombination. We are currently testing that prediction in the *lac* system. An association between double-strand break-repair recombination and mutagenic DNA synthesis was demonstrated in growing (unstressed) yeast cells.[27,54] This supports the idea that recombination-dependent mutational mechanisms such as that being described in the *E. coli lac s*ystem are relevant to mutation in other organisms.

Mutation in cells experiencing Lac^+ stationary-phase mutation is clearly not confined to the F′ because mutations are detected throughout the genome.[59] However, the mechanism of chromosomal mutation remains to be determined. We will consider possible mechanisms of chromosomal stationary-phase hypermutation.

POSSIBLE MECHANISMS OF GENOME-WIDE HYPERMUTATION DURING STATIONARY PHASE

First, hypermutation on both the F′ and the chromosome could occur via the RecABC-dependent mechanism described for *lac* stationary-phase mutation on the F′, including the requirement for F′ transfer activities for optimal mutation. Alternatively, the mutational mechanism may be the same except that double-strand ends for RecBCD are generated differently on the F′ than on the chromosome. A third possibility is that hypermutation of chromosomal loci occurs by a mechanism different from that described for stationary-phase mutation on the F′.

Hypothesis 1. Chromosomal Hypermutation Occurs via Exactly the Same Mechanism as That for Stationary-Phase Lac^+ Mutation on the F′

In this model, hypermutation of chromosomal loci is related to that on the F′ by a requirement for covalent linkage between the F′ and the chromosome (Hfr formation). Hfr formation would place the F′ origin of transfer, or whatever feature of F DNA brings F′

transfer functions to bear on mutation,[16,18] in *cis* to the bacterial chromosome, allowing RecABC-dependent mutation of adjacent chromosomal DNA. A test of one model of this type will be described.

Another possibility in which hypermutation would depend on the F′ is that an F′-encoded function might act on chromosomal sites. This would not require covalent attachment of the F and the chromosome. A chromosomal hypermutation mechanism that requires an F-encoded function might be similar to or different from the F′ Lac$^+$ mutational mechanism in other respects.

Does Mutation of Chromosomal Sites Correlate with Hfr Formation?

We have tested one version of the idea that Hfr formation allows chromosomal stationary-phase hypermutation. Our previous work on chromosomal mutations associated with Lac$^+$ reversion[59] provides a source of cells that carry unselected chromosomal mutations acquired during Lac$^+$ stationary-phase mutation. Here, we use Lac$^+$ stationary-phase mutant isolates carrying unselected chromosomal mutations[59] in the *upp* gene (conferring 5-fluorouracil resistance, 5FU^R) to ask whether acquisition of an unselected chromosomal mutation is correlated with Hfr formation. If so, this would support the idea that an identical mechanism creates both F′ and, by formation of an Hfr, chromosomal stationary-phase mutations.

To test this hypothesis, we determined the frequency of transfer of an F′ marker and multiple chromosomal markers in conjugational crosses using Lac$^+$ isolates, 5FU^R Lac$^+$ isolates, and the Lac$^-$ parental strain as donors. Multiply auxotrophic and fermentation-defective isogenic *rec*$^+$ and *recA* strains were used as recipients. The Lac$^-$ parental strain[6] and its Lac$^+$ stationary-phase mutant derivatives[59] carry a *pro*$^+$ F′ with the *lac* +1 frameshift allele (reverted in the case of Lac$^+$ derivatives) and a chromosomal deletion of the *lac-pro* region. The data are shown in TABLES 1 and 2 and displayed graphically in FIGURE 1.

First, as expected for transfer of a *pro*$^+$ F′, the frequency of transfer of proline prototrophy (Pro$^+$) is higher than that for any other marker tested for the Lac$^-$ parental strain and its Lac$^+$ derivatives (TABLE 1 and FIG. 1A). Also, the frequency of transferring Pro$^+$ in a 200-minute mating is unaffected by the presence of a *recA* mutation in the recipient as expected for an F′ which does not require recombination with the chromosome to form a stable transconjugant.[9] If the donors were stable Hfrs, then the acquisition of Pro$^+$ would depend on RecA because the recipients would become Pro$^+$ by homologous recombination. Thus, the cells in donor cultures appear to be largely F′-containing and not Hfrs. At the 20-minute time point, transfer is slightly decreased in the *recA* strain, suggesting that 20 minutes were insufficient for transfer of the entire F′ molecule in these experiments.

Second, the Lac$^-$ parental strain, Lac$^+$ isolates, and 5FU^R Lac$^+$ isolates all displayed much lower and similar frequencies for transfer of several chromosomal markers than for transfer of Pro$^+$ on the F′ (TABLE 1, FIG. 1B-E). The detectable transfer depends largely on *recA* (TABLES 1 and 2, FIG. 1B-E), implying that the markers are being transferred by an Hfr rather than an F′. Two control Hfr strains show *recA*-dependent transfer of chromosomal markers (TABLES 1 and 2 and FIG. 1C–E). An F$^+$ control strain gives low but detectable, and only partially *recA*-dependent, transfer of chromosomal markers. This suggests that perhaps some F′ molecules have formed in this donor. Thus, the F′ (and F$^+$ control)

TABLE 1. Frequency of Transfer of Chromosomal Markers[a]

Marker	Length of Mating (min)	No. of Transconjugants per Donor Cell (mean ± SE)[c]					
		Lac^{-h} ($n = 6$)[b]	Lac$^+$ Revertants[h] ($n = 9$)[b,d]	Unselected *upp* Mutants[h] ($n = 10$)[b]	Hfr strain with *oriT* Close to Selected Marker[h] ($n = 2$)[b,e]	Hfr strain with *oriT* Far from Selected Marker[h] ($n = 2$)[b,e]	F^{+h} ($n = 2$)[b,e]
Pro$^+$	20	1.6×10^{-3}	1.9×10^{-3}	1.7×10^{-3}	nd[i]	nd[i]	2.7×10^{-8}[f]
		(4.4×10^{-4})	(4.3×10^{-4})	(5.6×10^{-4})			
	200	5.2×10^{-2}	3.8×10^{-2}	2.2×10^{-2}	nd[i]	nd[i]	2.2×10^{-6}
		(3.6×10^{-3})	(6.0×10^{-3})	(7.2×10^{-3})			(1.7×10^{-7})
Arg$^+$	20	3.3×10^{-6}	5.0×10^{-6}	4.8×10^{-6}	nd[i]	nd[i]	2.6×10^{-8}[f]
		(4.0×10^{-7})	(3.9×10^{-6})	(3.3×10^{-7})			
	200	6.2×10^{-5}	8.6×10^{-5}	6.1×10^{-5}	nd[i]	nd[i]	$<1.3 \times 10^{-8}$[f]
		(2.7×10^{-6})	(3.7×10^{-5})	(4.0×10^{-6})			
Xyl$^+$	20	6.2×10^{-7}	5.6×10^{-7}	3.0×10^{-7}	2.5×10^{-4}	nd[i]	$<1.3 \times 10^{-8}$[g]
		(7.8×10^{-8})	(3.3×10^{-7})	(6.5×10^{-8})	(3.6×10^{-5})		
	200	3.7×10^{-5}	2.7×10^{-5}	1.8×10^{-5}	5.8×10^{-3}	nd[i]	4.5×10^{-7}
		(3.7×10^{-6})	(2.8×10^{-6})	(3.0×10^{-6})	(7.9×10^{-4})		(2.7×10^{-8})
His$^+$	20	5.5×10^{-7}	4.8×10^{-7}	4.3×10^{-7}	2.0×10^{-5}	1.3×10^{-5}	1.3×10^{-7}[f]
		(7.5×10^{-8})	(2.5×10^{-7})	(5.6×10^{-8})	(2.4×10^{-6})	(1.8×10^{-6})	
	200	5.2×10^{-6}	4.5×10^{-6}	2.8×10^{-6}	1.9×10^{-4}	7.2×10^{-5}	2.0×10^{-6}
		(1.5×10^{-7})	(5.0×10^{-7})	(3.0×10^{-7})	(3.1×10^{-5})	(1.1×10^{-6})	(8.4×10^{-7})
Gal$^+$	20	1.8×10^{-7}	6.0×10^{-7}	3.8×10^{-7}	nd[i]	2.6×10^{-6}	7.8×10^{-8}[f]
		(3.6×10^{-8})	(4.8×10^{-7})	(1.6×10^{-8})		(1.8×10^{-7})	
	200	5.0×10^{-6}	3.6×10^{-6}	2.4×10^{-6}	nd[i]	5.5×10^{-5}	1.2×10^{-6}
		(2.9×10^{-7})	(5.4×10^{-7})	(5.1×10^{-7})		(2.8×10^{-6})	(4.7×10^{-7})

[a]Transfer frequency was determined in conjugational crosses as follows: Equal volumes of mid-log phase donor and saturated AB1157 [*thr-1 ara-14 leuB6 lacY1 supE44 tsx-33* λ-rac-*xyl-5 gsr′ galK2 hisG4 rpsL31 mtl-1 argE3 thi-1* Δ(*gpt-proA*)*62 rfbD1 mgl-51 kdgK51* ([2])] (so that recipient would be in excess) were mixed in LBH 0.2% glucose and incubated for either 20 or 200 minutes at 37°C without shaking. Mating mixes were then washed in minimal M9 salts, and 10-μl spots of dilutions were placed on selective media. Selective media consisted of M9 thiamine 0.2% glucose plus streptomycin (100 μg/ml), leucine, threonine, and with or without histidine, proline, or arginine to select for histidine, proline, or arginine prototrophy, respectively. Media selective for the ability to ferment xylose and galactose contained those sugars, respectively, as carbon source. Donor strains were diluted and plated to determine the number of viable cells per milliliter. Plates were incubated overnight at 37°C and colonies were counted. The frequency of transfer was calculated as the number of transconjugants per donor cell. All media used are as described in ref. 36. All values shown in TABLES 1 and 2 are from crosses done in parallel in one large experiment.

[b]n, number of independent donor isolates tested. Each Lac$^+$ and 5FU^R Lac$^+$ isolate is an independently isolated stationary-phase Lac$^+$ mutant.

[c]SE, one standard error of the mean; except for Hfr and F$^+$ controls where the error given is calculated as the standard deviation but with only two values and thus represents a measure of variance.

[d]One of 10 Lac$^+$ isolates tested appeared to be mating-deficient, because it gave no transconjugants at all except in the Pro$^+$ 200′ selection. This isolate was therefore excluded from all calculations, making $n = 9$.

[e]Values here are an average of 2 values. Instead of a standard error of the mean, the variance is calculated as a standard deviation but with 2 data.

[f]In these cases only 1 donor isolate yielded transconjugants, and the average given is of the frequency for that isolate and a frequency of 0 for the others.

[g]< values were calculated as if one transconjugant had been observed in cases in which none were.

[h]Genotypes of donors: Lac$^-$ cells [FC40[6]: *ara* Δ(*lac-pro*)$_{XIII}$ Rif^R *thi-1* F′ *lacI33*Ω*lacZ proAB*$^+$]. Lac$^+$ stationary-phase mutants of FC40 and Lac$^+$ stationary-phase mutants carrying unselected *upp* mutations (conferring resistance to 5-fluoro-uracil, 5FU^R) are described by Torkelson *et al.*[59] The F$^+$ donor is R881 [*lac-3350* IN(*rrnD-rrnE*)1]. The Hfr donor control BW6175 should transfer Xyl$^+$ early and His$^+$ later. BW5660 should transfer His$^+$ early and Gal$^+$ later. See refs. 61 and 32 for Hfr donor genotypes and positions.

[i]nd, not determined.

TABLE 2. Frequency of Transfer of Chromosomal Markers into a *recA* Recipient[a]

Marker	Length of Mating (min)	No. of Transconjugants per Donor Cell (mean ± SE)[c]					
		Lac$^-$ [h] ($n = 6$)[b]	Lac$^+$ Revertants[h] ($n = 9$)[d]	Unselected *upp* Mutants[h] ($n = 10$)	Hfr Strain: *oriT* Close Selected Marker ($n = 2$)[h,e]	Hfr Strain: *oriT* Far from Selected Marker ($n = 2$)[e,h]	F$^+$ ($n = 2$)[e,h]
Pro$^+$	20	3.3×10^{-5} (4.1×10^{-6})	2.1×10^{-5} (4.5×10^{-6})	1.2×10^{-5} (3.3×10^{-6})	nd[i]	nd[i]	1.0×10^{-7}[f]
	200	5.3×10^{-2} (4.2×10^{-3})	3.0×10^{-2} (7.0×10^{-3})	2.0×10^{-2} (1.3×10^{-3})	nd[i]	nd[i]	4.1×10^{-7}[f]
Arg$^+$	20	$<1 \times 10^{-9}$[g]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	nd[i]	nd[i]	$<1.3 \times 10^{-8}$[g]
	200	$<1 \times 10^{-9}$[g]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	nd[i]	nd[i]	$<1.3 \times 10^{-8}$[g]
Xyl$^+$	20	$<1 \times 10^{-9}$[g]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	$<2.0 \times 10^{-8}$[g]	nd[i]	$<1.3 \times 10^{-8}$[g]
	200	2.3×10^{-7} (2.4×10^{-8})	2.2×10^{-7} (9.3×10^{-8})	1.3×10^{-7} (2.4×10^{-8})	2.0×10^{-6} (1.4×10^{-7})	nd[i]	$<1.3 \times 10^{-8}$[g]
His$^+$	20	$<1 \times 10^{-9}$[g]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	$<1.1 \times 10^{-8}$[g]	6.2×10^{-7} (2.4×10^{-7})	$<1.3 \times 10^{-8}$[g]
	200	8.7×10^{-8}[f]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	6.1×10^{-7} (1.6×10^{-7})	1.2×10^{-7}[f]	$<1.3 \times 10^{-8}$[g]
Gal$^+$	20	$<1 \times 10^{-9}$[g]	$<4.3 \times 10^{-10}$[g]	$<2.3 \times 10^{-10}$[g]	nd[i]	$<1.1 \times 10^{-8}$[g]	$<1.3 \times 10^{-8}$[g]
	200	1.2×10^{-8}[f,j]	5.0×10^{-9}[j]	8.7×10^{-9}[j]	nd[i]	3.0×10^{-7} (1.1×10^{-7})	$<1.3 \times 10^{-8}$[g]

[a] As in TABLE 1 except that the recipient strain is an isogenic *recA* derivative of AB1157 [carrying *Δ(srlR-recA)304*].

[b–i] As in TABLE 1.

[j] In these cases only, either 2 or 3 of the donor isolates gave transconjugants and an error was not calculated.

donor populations may be mixed with regard to F status. Some Hfr cells may be present among the F′ cells, and these could mediate transfer of chromosomal markers.

Third, the patterns of marker transfer among the Lac^+ and $5FU^R$ Lac^+ descendents of the hypermutable subpopulation are very similar to their Lac^- parent. Therefore, a stable change in Hfr status is not correlated with either the hypermutable state or the acquisition of unselected chromosomal mutations.

The frequency and *recA*-dependence of transfer of Pro^+ were also determined for three independent cultures of several Lac^+ derivatives. (Data in TABLES 1 and 2 represent averages of transfer frequencies of single cultures of independently isolated Lac^+ mutants.) The results in TABLE 3 indicate that independent isolates of Lac^+ derivatives behave similarly.

Transitory Hfr cells exist in a population derived from an F′ parent,[40,41] and many characterized Hfrs are known to be unstable. Although we find that the Lac^- and Lac^+ donors are not stable Hfrs, a transient population of unstable Hfrs might give rise to chromosomal mutations during stationary-phase hypermutation. An interesting possibility is that the hypermutable subpopulation may in fact be a transient subpopulation of Hfr cells. Addi-

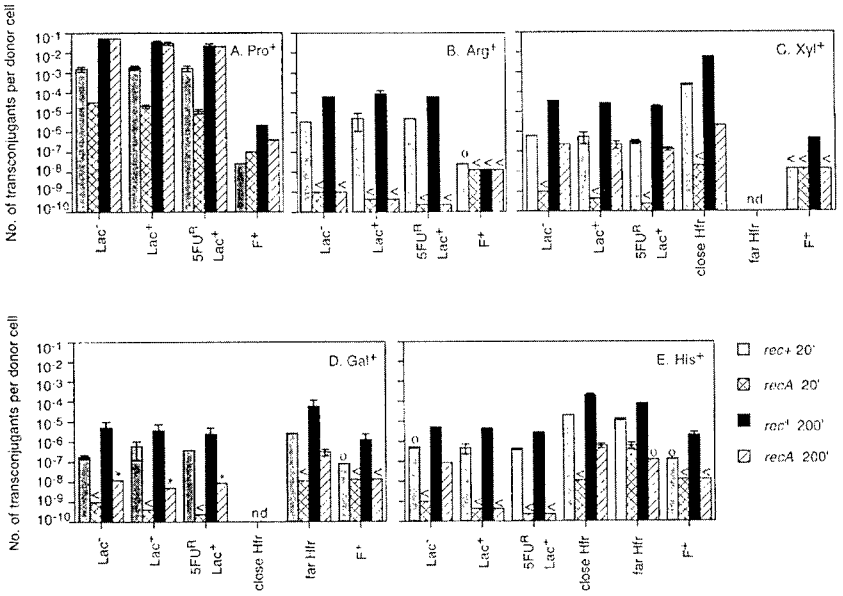

FIGURE 1. Frequency of conjugal transfer of episomal and chromosomal markers into rec^+ and *recA* recipients. Frequencies of transfer following either 20 minutes (20′) or 200 minutes (200′) of mating are shown. This is a graphic representation of the data presented in TABLES 1 and 2. Please see those tables for additional information. Pro^+ **(A)** is transferred from the F′ and the remaining markers (**[B]** Arg^+, **[C]**, Xyl^+, **[D]** Gal^+, and **[E]** His^+) are transferred from the chromosome. Where given, error bars represent 1 standard error of the mean. Values for which no error was calculated because only two or three isolates (of 10) gave transconjugants are marked with an asterisk. "Less than" values (see TABLE 1) are marked with "<."

TABLE 3. RecA-Dependence of Transfer Determined for Multiple Isolates of Lac$^+$ Derivatives[a]

			No. of Transconjugants per Donor Cell (mean ± SE)[d]								
				Lac$^+$ Revertants[c]			Lac$^+$ Unselected *upp* Mutants[c]				
Marker	Recipient *rec* Genotype	Length of Mating (min)	Lac$^-$ ($n = 10$)[b]	SMR1140 ($n = 3$)[b]	SMR1142 ($n = 3$)[b]	SMR1146 ($n = 3$)[b]	#242 ($n = 3$)[b]	#314 ($n = 3$)[b]	#512 ($n = 3$)[b]	#514 ($n = 3$)[b]	F$^+$ ($n = 4$)[b]
Pro$^+$	*rec*$^+$	20	3.1×10^{-2} (9.3×10^{-3})	3.6×10^{-3} (1.3×10^{-3})	4.0×10^{-2} (1.8×10^{-2})	1.3×10^{-2} (2.1×10^{-3})	2.3×10^{-4} (4.9×10^{-5})	6.8×10^{-2} (2.7×10^{-2})	2.3×10^{-2} (4.0×10^{-3})	5.9×10^{-3} (2.3×10^{-3})	$<1.5 \times 10^{-6}$[e]
	recA	20	7.4×10^{-2} (4.2×10^{-3})	8.5×10^{-2} (2.5×10^{-4})	8.5×10^{-2} (5.9×10^{-4})	8.9×10^{-2} (1.0×10^{-2})	2.2×10^{-4} (1.2×10^{-5})	1.5×10^{-1} (1.5×10^{-2})	9.8×10^{-2} (6.1×10^{-3})	5.7×10^{-2} (1.2×10^{-2})	9.5×10^{-6}[f]
	rec$^+$	200	1.6×10^{0} (1.9×10^{-1})	1.6×10^{0} (5.7×10^{-1})	2.0×10^{0} (2.9×10^{-1})	1.2×10^{0} (1.5×10^{-1})	2.5×10^{-2} (5.8×10^{-3})	1.4×10^{0} 3.2×10^{-1})	9.6×10^{-1} (1.2×10^{-1})	1.1×10^{0} (3.2×10^{-1})	4.1×10^{-5}[f]
	recA	200	1.6×10^{0} (1.9×10^{-1})	2.2×10^{0} (7.2×10^{-1})	3.0×10^{0} (5.6×10^{-1})	2.4×10^{0} (1.9×10^{-1})	1.2×10^{-1} (1.1×10^{-2})	2.6×10^{0} (2.2×10^{-1})	2.6×10^{0} (3.8×10^{-1})	1.6×10^{0} (4.3×10^{-1})	2.1×10^{-4} (4.1×10^{-5})

[a]Transfer frequencies were determined as described in TABLE 1, except that these values are from crosses done in parallel in a second large experiment. Recipients and donor genotypes are described in TABLES 1 and 2.

[b]*n*, number of independent isolates tested.

[c]Each value for the Lac$^+$ derivatives is derived from three independent isolates of a given Lac$^+$ stationary-phase mutant. SMR1140, 1142, and 1146 are independent Lac$^+$ stationary-phase revertants (R.S. Harris, unpublished results) and #242, 314, 512 and 514 are independent *upp* Lac$^+$ mutants isolated from a stationary-phase mutation assay.[59]

[d]SE, one standard error of the mean.

[e]< values were calculated as if one transconjugant had been observed in cases in which none were.

[f]In these cases only 1 donor isolate yielded transconjugants and the average given is of the frequency for that isolate and a frequency of 0 for the others.

tional experiments would be needed to address the possible role of unstable Hfrs in the generation of chromosomal stationary-phase mutations. We are pursuing other lines of study that will reveal direct or indirect roles of the F′ in chromosomal stationary-phase mutation.

Hypothesis 2. Chromosomal Hypermutation Is Initiated Independently of the F and Then Proceeds by the Same Mechanism

In this hypothesis, chromosomal mutations that occur during stationary-phase mutation (e.g., those detected in Lac$^+$ stationary-phase mutants) are independent of the F′ in origin, yet occur by the same RecBCD-dependent mechanism. This requires an initiating double-strand break or end (which on the F′ is hypothesized to be created by processing of a nick at *oriT*[49]). A DSB or DSE might result from oxidative[5] or other DNA damage,[48] a collapsed[28] or stalled replication fork,[25,34,48] or be created by a programmed DNA cleavage event.[48] Once RecBCD gains access to the DNA at the DSE the mechanism proceeds as at *lac* on the F′.

We are testing this hypothesis by determining whether recombination-dependent mutation occurs at various sites in the chromosome in the absence of an F episome. The existence of associated chromosomal mutations suggests that chromosomal sites are susceptible to the same recombination-dependent mechanism. However, if the *lac* +1 frameshift allele is placed at the chromosomal *lac* locus, it does not experience detectable RecA-dependent mutation.[16,42] One "cold" site for mutation does not exclude the entire chromosome from recombination-dependent mutation. "Hot" and "cold" regions for chromosomal mutation exist.[47,59] Proximity to a double-strand end (RecBCD access point) is one property that could influence whether a given site is hot or cold.[47,52] In this model, the F′ is hot due to the presence of a nicking site, *oriT*.[47,49,52]

Hypothesis 3. Chromosomal Stationary-Phase Hypermutation Occurs by a Different Mechanism

This is a more complex model in which multiple mutational processes would have to occur coincidentally during stationary-phase hypermutation. This model is not supported by experiments demonstrating that chromosomal mutation increases in hyper-recombinagenic strains that have elevated recombination-dependent mutation on the F′, suggesting recombinational involvement in chromosomal mutation (H. Bull, G.J. McKenzie, and S.M. Rosenberg, unpublished results). However, other mutational mechanisms do occur in stationary-phase cells. Other examples of mutation in nongrowing or slowly growing cells under selection are independent of *recA*[4,19,21,42] and thus occur by a mechanism different from that described for Lac$^+$ mutation on the F′.

The SOS Response

The SOS response to DNA damage may be a component of multiple pathways of mutation in stationary phase. The SOS response is induced by DNA damaging agents and

results in increased levels of DNA recombination and repair proteins and mutagenesis.[60] Any SOS-dependent mutagenic pathway would also be RecA-dependent because RecA is required for SOS induction.[60] SOS induction is required for full levels of *lac* stationary-phase mutation on the F′.[6,22] Although it was thought that SOS induction served merely to provide increased levels of RecA,[6] this conclusion was based on experiments with an improperly constructed strain (as mentioned in ref. 17). It is now clear that an SOS-regulated function other than, or in addition to RecA, is required for efficient stationary-phase mutation.[22] The SOS response, along with the signaling molecule cAMP, is required for chromosomal mutagenesis described in aging *E. coli* colonies.[56] The genetic requirements for this mutagenic process are similar to those of *lac* stationary-phase mutation, but with a few important differences. The SOS requirement is partial in one case[6,22] and complete in the other.[55] In addition, RecBC, which is essential for stationary-phase *lac* reversion,[25] is only partially required for mutagenesis in aging colonies.[55] The mutagenesis in aging colonies[55] and in the *lac* frameshift assay system[6] is independent of UmuC, an SOS-induced protein that, with UmuD, mediates error-prone DNA synthesis during the SOS response.[60] Other SOS-dependent pathways to mutation are possibilities for alternative routes to chromosomal hypermutation in stationary-phase cells.

Alternatively, chromosomal hypermutation in stationary-phase cells may be independent of the SOS response and instead rely on other mutational pathways that remain to be discovered. Mechanistic information is available for only a few instances of mutation in stationary-phase cells.[20,47,55,56,58] Various mechanisms may be discovered as this field is explored.[11] The role of recombination and the SOS response in chromosomal hypermutation will be revealed by examining mutation at chromosomal sites. This will distinguish between the three broad categories of possibilities discussed here for the mechanism of genome-wide hypermutation.

SUMMARY

Although the mechanism of chromosomal hypermutation in stationary phase remains to be determined, one specific model in which stable Hfr formation is a prerequisite for F-dependent mutation of chromosomal loci has been ruled out. Further studies will reveal the mutational mechanism responsible for chromosomal hypermutation including the role, if any, of the F. Genome-wide hypermutation in stationary phase represents a powerful opportunity to alter the genome in response to starvation or stressful environmental conditions. The resulting variation includes mutations that have immediate benefit for the cell, allowing survival and growth in suboptimal conditions, that is, adaptive mutations. Such generation of variation has important implications for bacterial evolution. The recombination-dependent mechanism itself appears broadly relevant to mutation formation in other types of nondividing or slowly growing cells such as those that give rise to cancers, to drug resistance in the presence of mitosis-inhibiting chemotherapeutic drugs, and to resistance to antibiotics that kill growing cells.

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[NOTE ADDED IN PROOF: The evidence cited for downregulation of mismatch repair in human cells[44] has been retracted (Meuth, M., B. Richards & B. Schneider. The conditional mutator phenotype in human tumor cells: Correction. Science **283:** 639). Thus, stationary-phase mutation in *E. coli* is the only system, so far, in which good evidence exists,[24,31] indicating that mismatch repair activity can be modulated in response to environmental conditions.]

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Evidence That Stationary-Phase Hypermutation in the *Escherichia coli* Chromosome Is Promoted by Recombination

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ABSTRACT

Adaptive (or stationary-phase) mutation is a group of phenomena in which mutations appear to occur more often when selected than when not. They may represent cellular responses to the environment in which the genome is altered to allow survival. The best-characterized assay system and mechanism is reversion of a *lac* allele on an F′ sex plasmid in *Escherichia coli*, in which the stationary-phase mutability requires homologous recombination functions. A key issue has concerned whether the recombination-dependent mutation mechanism is F′ specific or is general. Hypermutation of chromosomal genes occurs in association with adaptive Lac$^+$ mutation. Here we present evidence that the chromosomal hypermutation is promoted by recombination. Hyperrecombinagenic *recD* cells show elevated chromosomal hypermutation. Further, *recG* mutation, which promotes accumulation of recombination intermediates proposed to prime replication and mutation, also stimulates chromosomal hypermutation. The coincident mutations at *lac* (on the F′) and chromosomal genes behave as independent events, whereas coincident mutations at *lac* and other F-linked sites do not. This implies that transient covalent linkage of F′ and chromosomal DNA (Hfr formation) does not underlie chromosomal mutation. The data suggest that recombinational stationary-phase mutation occurs in the bacterial chromosome and thus can be a general strategy for programmed genetic change.

STATIONARY-PHASE (or adaptive) mutations occur in nondividing or slowly growing cells exposed to a nonlethal selection (reviewed by DRAKE 1991; FOSTER 1993; HALL 1993; SYMONDS 1993; ROSENBERG *et al.* 1994; ROSENBERG 1997; LOMBARDO *et al.* 1999a; LOMBARDO and ROSENBERG 1999). They differ from spontaneous growth-dependent mutations, which occur in dividing cells, before exposure to an environment selective for the mutation, and randomly in the genome (*e.g.*, LURIA and DELBRÜCK 1943). In some assay systems for stationary-phase mutation, the mutations may occur preferentially in genes whose functions are selected (WRIGHT *et al.* 1999). In the system used here, genome-wide hypermutability appears to underlie adaptive mutations (*i.e.*, those mutations selected) and produce non-adaptive mutations concurrently (TORKELSON *et al.* 1997; postulated by HALL 1990; NINIO 1991), although nonrandomness in the form of "hot" and "cold" sites for the mutation has been documented (ROSENBERG 1997; TORKELSON *et al.* 1997). Stationary-phase mutations form via multiple different mechanisms, some of which clearly differ from spontaneous growth-dependent mutation (MAENHAUT-MICHEL and SHAPIRO 1994; HALL 1995; MAENHAUT-MICHEL *et al.* 1997; ROSENBERG 1997; TADDEI *et al.* 1997; WRIGHT *et al.* 1999). The molecular mechanisms of mutation in nongrowing and slowly growing cells under stress provide important models for evolution of microbes in real-world, stressful environments, for mutations that confer resistance to antibiotics and chemotherapeutic drugs, and for mutations that initiate cancer in cells that are not growing actively. Elucidation of mechanisms of mutation in response to selection is modifying core concepts in biological evolution and development (*e.g.*, CAIRNS *et al.* 1988; CULOTTA 1994; THALER 1994; SHAPIRO 1997; PENNISI 1998; CAPORALE 1999). Understanding these mechanisms will illuminate their roles in evolution, development, cancer formation, and genome structure and function, all of which may be underpinned by such dynamic mutational processes.

The best-studied assay for stationary-phase mutation uses *Escherichia coli* cells carrying a revertible *lac* frameshift allele on an F′ sex plasmid and no *lac* genes in the chromosome (CAIRNS and FOSTER 1991). Growth-dependent Lac$^+$ revertants, carrying mutations formed prior to plating on lactose minimal medium, appear after about 2 days of incubation on lactose plates. Additional Lac$^+$ mutant colonies appear each day for several days and these carry mutations formed during starvation on the lactose medium (MCKENZIE *et al.* 1998; stationary-phase mutations). The stationary-phase mutations form via a unique molecular mechanism that differs from growth-dependent Lac$^+$ mutations as follows:

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1. Homologous recombination functions *recA*, *recB*, *ruvA*, *ruvB*, and *ruvC* are required for stationary-phase, but not growth-dependent Lac$^+$ mutation (Harris *et al.* 1994, 1996; Foster *et al.* 1996).
2. Because RecBCD loads onto DNA only at double-strand DNA ends (DSEs), DSEs are implicated as molecular intermediates in the mutagenic process (Harris *et al.* 1994).
3. Formation of stationary-phase Lac$^+$ mutations requires F-encoded transfer functions (Foster and Trimarchi 1995a; Galitski and Roth 1995), but not actual F plasmid transfer (Foster and Trimarchi 1995a,b; Radicella *et al.* 1995; Rosenberg *et al.* 1995). One possible explanation for this requirement is that the single-strand nick produced at the transfer origin by transfer (Tra) proteins develops into a double-strand break (DSB) and that this is the major DSB source on the F plasmid (Kuzminov 1995; Rosenberg *et al.* 1995).
4. Stationary-phase Lac$^+$ mutations are nearly all -1 deletions in small mononucleotide repeats, whereas the growth-dependent Lac$^+$ mutations are heterogeneous (Foster and Trimarchi 1994; Rosenberg *et al.* 1994).
5. The stationary-phase mutations are attributable to DNA polymerase errors made by the major replicative polymerase, PolIII (Foster *et al.* 1995; Harris *et al.* 1997a).
6. These errors persist under conditions of insufficient postreplicative mismatch repair (MMR) activity (Longerich *et al.* 1995), during which the MutL MMR protein becomes limiting (Harris *et al.* 1997b, 1999a).

The recombination-dependent stationary-phase mutations are proposed to result from DNA replication at sites of DSB repair via homologous recombination (Harris *et al.* 1994; reviewed by Rosenberg 1997; Lombardo and Rosenberg 1999) as follows: DSBs are suggested to occur during the stress of starvation on lactose medium (see Harris *et al.* 1994; Kuzminov 1995; Rosenberg *et al.* 1995, 1996; Bridges 1997; Seigneur *et al.* 1998, for suggestions on how DSBs could form). RecBCD loads onto DSEs and digests and unwinds the DNA, producing single-stranded DNA ends, which are used by RecA protein for strand invasion of a homologous DNA molecule (Figure 1). The D loops are proposed to prime DNA replication (Harris *et al.* 1994; Kogoma 1997; see Liu *et al.* 1999; Motamedi *et al.* 1999) using DNA PolIII (Foster *et al.* 1995; Harris *et al.* 1997a). Polymerase errors are suggested to persist due to transient MMR deficiency (Longerich *et al.* 1995; Harris *et al.* 1997b). These become Lac$^+$ (and other) mutations.

The "adaptive" nature of these mutations can be accounted for by a modification of Hall's proposal in which adaptive mutations arise in a hypermutable subpopulation of cells exposed to selection (Hall 1990; see Ninio 1991). Both nonadaptive and adaptive (Lac$^+$) mutations are proposed to form. However, the nonadaptive mutations might not be readily apparent in the main population either because of their low number or due to death of mutant cells that had not also acquired an adaptive mutation. This model was supported in the Lac system by the demonstrations of high frequencies of mutation at multiple sites, genome-wide, in Lac$^+$ colony formers, but not in the main population of (Lac$^-$) cells exposed to selection (Torkelson *et al.* 1997; Rosche

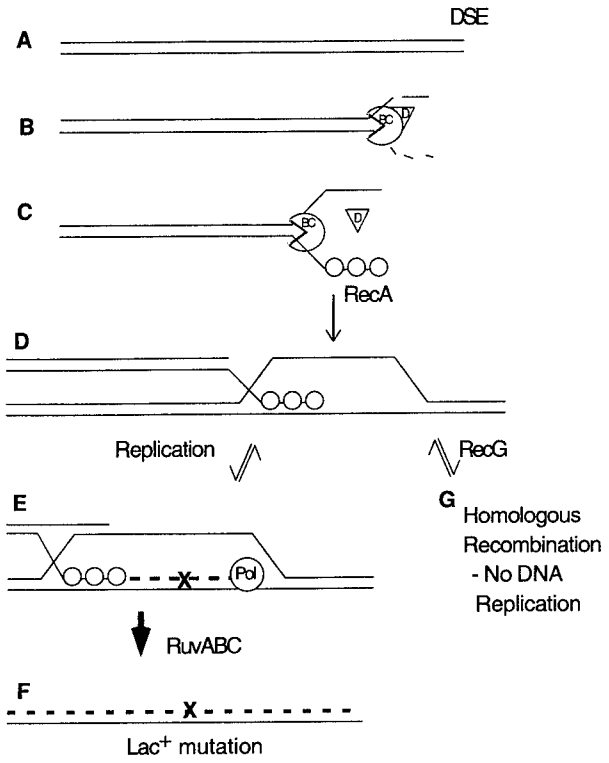

Figure 1.—A model for formation of recombination-dependent stationary-phase Lac$^+$ mutations. (A) A double-strand end (DSE) is proposed to occur [*e.g.*, via processing of a Tra-dependent nick (Rosenberg *et al.* 1995), disintegration (Kuzminov 1995) or stalling (Rosenberg *et al.* 1996; Michel *et al.* 1997; Seigneur *et al.* 1998) of a replication fork, or other mechanism (*e.g.*, Rosenberg 1994, 1997; Bridges 1997)]. (B) The DSE is processed by the RecBCD enzyme, creating single-stranded DNA ends (C) that become bound by RecA (small circles), which catalyzes invasion of a homologous duplex to produce a displacement loop (D). (E) The invading strand (in this example a 3′-ended single strand) serves as a primer and loading site for the replicative DNA polymerase PolIII (Liu *et al.* 1999). Errors produced by PolIII (X) may remain uncorrected due to a transient deficiency in methyl-directed mismatch repair (Longerich *et al.* 1995; Harris *et al.* 1997b). The error becomes genetically fixed giving a Lac$^+$ mutation (X in F). An alternate outcome of intermediate D is that strand invasion (perhaps from 5′-ended single-strand invasions, which cannot serve as a primer) leads to (G) homologous recombination with no associated DNA replication (Harris *et al.* 1996; Rosenberg and Motamedi 1999; Motamedi *et al.* 1999). (- - -) Newly synthesized DNA.

and FOSTER 1999). These unselected mutations appear to form concurrently with the Lac$^+$ adaptive mutations (not during growth of the Lac$^+$ colony) as seen by their representation in all cells (not sectors) of the Lac$^+$ mutant colonies.

Although it is clear that (1) a fundamentally different mutation mechanism generates the Lac$^+$ stationary-phase mutations, (2) the cells engaging in this mechanism are differentiated [transiently mismatch-repair deficient (LONGERICH *et al.* 1995; HARRIS *et al.* 1997b) and comprising a small hypermutable subpopulation (TORKELSON *et al.* 1997)], and also (3) chromosomal genes are mutated concurrently (TORKELSON *et al.* 1997; ROSCHE and FOSTER 1999), the possible relevance of the recombination-dependent stationary-phase mutation mechanism to mutation in the bacterial chromosome has been controversial (*e.g.*, FOSTER and TRIMARCHI 1995a,b; GALITSKI and ROTH 1995, 1996; RADICELLA *et al.* 1995; PETERS *et al.* 1996; BENSON 1997, discussed below). The issue underlying this question is whether the recombinational stationary-phase mutation mechanism affects the bacterial genome in general.

Recombination is a hallmark of this novel mutation mechanism. Here, we test the role of recombination in hypermutation of chromosomal genes that occurs concurrently with adaptive Lac$^+$ reversion. We find that two recombination-altered alleles, both of which promote recombination-dependent stationary-phase mutation at *lac* (on the F′), also promote concurrent hypermutation of chromosomal genes. The data imply that recombination-dependent stationary-phase mutation is not strictly an F-plasmid-specific mechanism, but rather is a mechanism for genetic change at multiple sites throughout the genome. We observe that mutations at *lac* and chromosomal sites occur as independent events, supportive of the idea that these sites are not joined covalently (as an Hfr) at the time of mutation. In contrast, mutation of *lac* and another F′-borne site does not appear to be independent. The data support the idea that recombination-dependent stationary-phase mutation is a mechanism for genetic change at multiple sites throughout the genome and thus may be a general response to stress and a strategy for evolution.

MATERIALS AND METHODS

***E. coli* strains:** A strain unable to revert to Lac$^+$ was used to scavenge carbon sources other than lactose (CAIRNS and FOSTER 1991). All other strains are derived from FC40 (CAIRNS and FOSTER 1991), which carries a large chromosomal deletion of the *lac* operon and neighboring genes, and an F′ sex plasmid carrying genes in the *lac* and *proAB* region. The *lac* allele on the F′ has a translational fusion of *lacI* with *lacZ* and a +1 frameshift mutation in *lacI* which is polar on *lacZ*. The *recD* derivative is SMR582 carrying *recD1903::*Tn*10miniTet* (HARRIS *et al.* 1994). The *recG* derivative is RSH316 carrying *recG258::*Tn*10miniKan* (HARRIS *et al.* 1996).

Mutation assays: Assays for Lac$^+$ stationary-phase mutation were performed as described (HARRIS *et al.* 1996). Assays for unselected secondary mutations were performed by replica-plating Lac$^+$ colonies, obtained in the Lac$^+$ assay after 5 days of incubation, to various indicator and selective media as described by TORKELSON *et al.* (1997). All presumptive secondary mutants were confirmed by streaking from the original Lac$^+$ colony (master colony) for single colonies on the appropriate indicator plate. The purity of Lac$^+$ colonies expressing fermentation mutations was determined by removing the master colonies with plugs of agar, suspending the cells in buffer, diluting, and spreading on minimal (M9 thiamine) lactose plates to obtain $\sim$ 100 Lac$^+$ colonies per plate. The resulting Lac$^+$ colonies were replica-plated to the appropriate MacConkey indicator medium and the numbers of fermentation-defective mutants and fermentation-competent colonies were determined. Typically, >80% of the secondary mutant colonies assayed were pure in that all Lac$^+$ colonies replica-plated were of the mutant phenotype. Mutations resulting in 5-fluorocytosine resistance (5FC^r) map to *codAB* or *upp* whereas mutations resulting in 5-fluorouracil resistance (5FU^r) map only to *upp* (TORKELSON *et al.* 1997). *upp* mutations were not useful in this study because we observed that both the *recD* and *recG* mutations are able to suppress the 5FU^r and 5FC^r phenotypes of a large (>80%) portion of the *upp* mutations (data not shown) and so only 5FC^r 5FU^s mutants were included. (Reconstruction experiments with known *upp* and *codA* mutations demonstrated that *upp* mutations that were suppressed for 5FU^r by *recD* and *recG* were also suppressed for 5FC^r. Thus all 5FC^r 5FU^s are at *codAB*.) 5FC^r colonies were tested for purity as described above. Typically, >80% of 5FC^r mutants identified in this manner were pure.

Unselected mutations in Lac$^-$ starved cells were assayed as described (TORKELSON *et al.* 1997). Plugs of agar were removed from between visible Lac$^+$ colonies each day and suspended in M9 buffer. Aliquots were spread on LBH and on MacConkey lactose plates and incubated. (This allowed detection of any Lac$^+$ colonies that were not yet visible and had been picked accidentally.) The resulting Lac$^-$ colonies (each derived from a Lac$^-$ cell starved on lactose) were screened for unselected mutations by replica-plating.

RESULTS

Strategy for measuring stationary-phase mutation in the bacterial chromosome: Chromosomal mutations coincident with Lac$^+$ stationary-phase mutation can be measured by replica-plating the Lac$^+$ stationary-phase mutant colonies to media selective for particular loss-of-function mutants or to color indicator media for fermentation-defective mutants (TORKELSON *et al.* 1997). The hypermutation of chromosomal genes is observed in the Lac$^+$ mutants only and not in the neighboring Lac$^-$ cells, which were also starved on lactose and then rescued, grown into colonies, and replica-plated. Such "Lac$^-$ stressed cell colonies" display low chromosomal mutation frequencies indistinguishable, in replica-plating assays, from Lac$^-$ cells never exposed to selection (TORKELSON *et al.* 1997; ROSCHE and FOSTER 1999, and below). Therefore, to score stationary-phase hypermutation of chromosomal genes, we obtained Lac$^+$ stationary-phase mutants to screen for the presence of additional mutations.

For three reasons, we infer that these additional chromosomal mutations occurred during transient, station-

ary-phase hypermutability and not during subsequent growth of the Lac$^+$ mutant cell into a colony: first, the Lac$^+$ colonies with additional mutations are mostly pure, not mixed (sectored), for the additional mutation, implying that the initial colony-forming cell carried the mutation (Torkelson *et al.* 1997; and shown again here, see materials and methods). Second, the Lac$^+$ mutants are not heritable mutator mutants (Longerich *et al.* 1995; Torkelson *et al.* 1997) and, third, they are not heritable stationary-phase mutator mutants (Rosenberg *et al.* 1998); thus they must have descended from a transiently mutable subpopulation. Lac$^-$ stressed cells, which show low frequencies of additional mutation (Torkelson *et al.* 1997; Rosche and Foster 1999, and below), make up the main population.

In recombination-defective strains, no Lac$^+$ stationary-phase mutants arise (Harris *et al.* 1994, 1996; Foster *et al.* 1996). Therefore we tested the role of recombination in chromosomal hypermutation using recombination-proficient cells with elevated stationary-phase Lac$^+$ mutation, *recD* and *recG* null mutants.

Rationale for use of *recD* and *recG* mutants: We tested whether two recombination gene defects that promote recombination-dependent stationary-phase mutation of *lac* on the F′ affect mutability of chromosomal genes in stationary phase. *recD* null alleles confer hyperrecombination (Chaudhury and Smith 1984; Amundsen *et al.* 1986; Biek and Cohen 1986; Thaler *et al.* 1989) and enhance stationary-phase mutation (Harris *et al.* 1994; Foster and Rosche 1999). Strains carrying *recG* null mutations are hypermutable in Lac$^+$ stationary-phase mutation (Foster *et al.* 1996; Harris *et al.* 1996), and several lines of evidence imply that RecG protein, which is a Holliday junction branch migration helicase (Whitby *et al.* 1994), interferes with those recombination intermediates that promote replication (Whitby *et al.* 1993; Al-Deib *et al.* 1996; Harris *et al.* 1996; McGlynn *et al.* 1997). Thus both *recD* and *recG* mutations increase numbers of the strand-exchange recombination intermediates thought to promote replication and both promote recombination-dependent stationary-phase mutation (see Figure 1).

***recD* and *recG* increase mutability of chromosomal genes in Lac$^+$ stationary-phase mutants:** Otherwise isogenic *rec*$^+$, *recD*, and *recG* strains were starved in parallel on lactose minimal medium. Following the fifth day of lactose selection, the Lac$^+$ colonies were replica-plated to appropriate indicator and selective media to reveal chromosomal loss-of-function mutants. Chromosomal mutations assayed were among those detected previously by Torkelson *et al.* (1997; and see materials and methods). The results of three separate experiments are presented in Table 1 and Figure 2. The *recD* null mutant showed approximately twice as many xylose (Xyl$^-$) and maltose (Mal$^-$) fermentation-defective mutations per Lac$^+$ colony as did *rec*$^+$ cells. The increased frequency of chromosomal mutation coincident with Lac$^+$ is similar to the increase in Lac$^+$ stationary-phase mutant frequency in the *recD* background (Figure 2). Because *recD* strains are hyperrecombinagenic (Chaudhury and Smith 1984; Amundsen *et al.* 1986; Biek and Cohen 1986; Thaler *et al.* 1989), these data suggest that the increased mutability of chromosomal loci and F-borne loci is due to recombination.

In the *recG* null strain, the frequency of Xyl$^-$ and Mal$^-$ mutations per Lac$^+$ mutant was, respectively, 4.6-fold and 6.0-fold higher than in the *rec*$^+$ control (Table 1 and Figure 2B). Also, fructose fermentation-defective (Fru$^-$) mutations, which previously (Torkelson *et al.* 1997) and here were so infrequent as to be undetectable in *rec*$^+$ cells, were detected in the *recG* strain (Table 1 and Figure 2B). Thus, loss of RecG increases the frequency of chromosomal mutations concurrent with Lac$^+$ stationary-phase mutation. The total increase (Xyl$^-$ plus Mal$^-$ plus Fru$^-$) is at least 5.7-fold (Table 1 and Figure 2B). Because RecG is a helicase that can unwind and abort recombination intermediates (Whitby *et al.* 1993; Al-Deib *et al.* 1996; Harris *et al.* 1996; McGlynn *et al.* 1997) and that inhibits recombination-dependent stationary-phase mutation (Foster *et al.* 1996; Harris *et al.* 1996), these data suggest that allowing recombination intermediates to enter a replication-promoting pathway in the RecG-deficient strain promotes chromosomal mutation, in agreement with the *recD* data (above).

recD and *recG* strains increase mutation at unselected chromosomal loci to an extent similar to their effect on Lac$^+$ colony formation (Figure 2). However, their effect on unselected mutations is smaller than that seen on Lac$^+$ colony formation. It may be that chromosomal genes cannot be mutated with the same efficiency as F′-borne genes, perhaps because of some sequence specificity of the mutation mechanism. The apparent difference in *recD* and *recG* could reflect varying susceptibility to mutagenesis for the chromosomal loci. We have observed hot and cold sites for unselected chromosomal mutation (Rosenberg 1997; Torkelson *et al.* 1997).

Increased mutation is limited to the hypermutable subpopulation: The increase in chromosomal mutations per Lac$^+$ stationary-phase mutant in *recD* and *recG* strains indicates that *recD* and *recG* loss increases mutability in cells that become Lac$^+$. We wished to know whether the elevated mutability is specific to the hypermutable subpopulation cells or whether loss of *recD* or *recG* increases the mutability of all cells exposed to starvation on lactose medium. Because of the large numbers of replica-plated colonies required to detect chromosomal mutations among Lac$^-$ starved cells [one to two orders of magnitude less frequent than among Lac$^+$ colonies, at 10^{-4} to 10^{-5} of the whole population (Torkelson *et al.* 1997)], we tested only the *recG* strain. Stationary-phase mutation is elevated so dramatically by *recG* that a *recG*-promoted increase in chromosomal mutability should be readily detectable even in the Lac$^-$ cells.

TABLE 1

Chromosomal mutations per Lac$^+$ adaptive mutant are increased in *recG* and *recD* cells

rec genotype	Mutant phenotype	No. of mutants among Lac$^+$ adaptive revertants (mutant colonies/Lac$^+$ colonies scored)[a] Expt. 1	Expt. 2	Expt. 3	Frequency of unselected chromosomal mutations/Lac$^+$ adaptive revertant[b] Mean ± SEM	
rec$^+$	Xyl$^-$	4/4080	2/4675	6/6253	7.9×10^{-4}	
	Mal$^-$	2/4080	0/4675	8/6253	$<6.6 \times 10^{-4}$	
	Fruc$^-$	0/4080	0/4675	0/6253	$<2.1 \times 10^{-4}$	
	Total[c]	6/4080	2/4675	14/6253	1.4×10^{-3}	(0.53×10^{-3})
recD	Xyl$^-$	5/3639	2/1038	4/8712	1.3×10^{-3}	
	Mal$^-$	6/3639	2/1038	15/8712	1.8×10^{-3}	
	Fruc$^-$	1/3639	0/1038	0/8712	$<4.5 \times 10^{-3}$	
	Total[c]	12/3639	4/1038	19/8712	3.1×10^{-3}	(0.49×10^{-3})
recG	Xyl$^-$	19/6427	14/3960	8/1834	3.6×10^{-3}	
	Mal$^-$	25/6427	19/3960	6/1834	4.0×10^{-3}	
	Fruc$^-$	2/6427	1/3960	1/1834	3.7×10^{-3}	
	Total[c]	46/6427	34/3960	15/1834	8.0×10^{-3}	(0.43×10^{-3})

[a] Three experiments were done with the three strains assayed in parallel.
[b] The mean of the frequencies for the three experiments (±1 SE).
[c] Total numbers for all phenotypes, each experiment.

To assay chromosomal mutations among Lac$^-$ stressed cells, those cells were recovered from between visible Lac$^+$ colonies after prolonged starvation and replated nonselectively to form colonies that were then replica-plated to screen for chromosomal mutants (see MATERIALS AND METHODS and TORKELSON *et al.* 1997). The data in Table 2 indicate that the low frequency of Mal$^-$ and Xyl$^-$ mutations per Lac$^-$ stressed cell colony [one to two orders of magnitude lower than per Lac$^+$ mutant (Table 2; also reported by TORKELSON *et al.* 1997)] is not increased detectably by the *recG* mutation. By contrast, Mal$^-$ and Xyl$^-$ mutations are increased per Lac$^+$ colony (Tables 1 and 2; Figure 2). These data imply that promotion of mutation by the absence of RecG is limited to the hypermutable subpopulation cells.

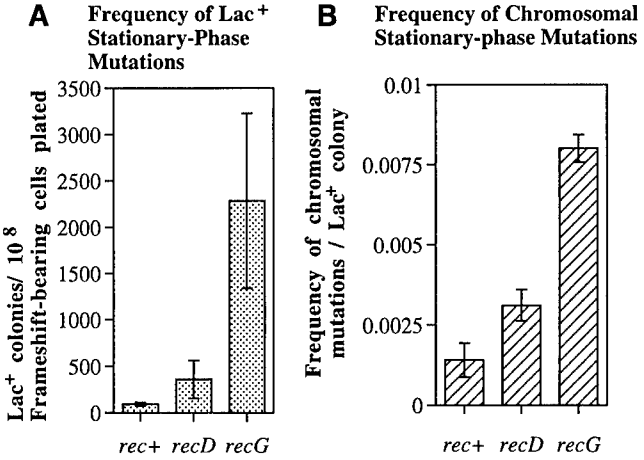

FIGURE 2.—(A) Lac$^+$ stationary-phase mutants accumulated over 5 days of selection in *rec*$^+$, *recD*, and *recG* strains. (B) The frequency of unselected chromosomal mutations (Mal$^-$, Xyl$^-$, and Fru$^-$) per Lac$^+$ stationary-phase mutant in *rec*$^+$, *recD*, and *recG* strains. The values are the mean of three separate experiments, with *rec*$^+$, *recD*, and *recG* tested in parallel each time [Table 1, total mean ± 1 SE (error bars)]. Values obtained for B are from the Lac$^+$ colonies reported in A.

Some condition present in the subpopulation, but not the main population, appears to be necessary for high levels of chromosomal stationary-phase mutation, as is the case for Lac$^+$ adaptive mutation. The condition that makes the subpopulation cells mutable could be the occurrence of DNA DSBs or DSEs at which recombination would occur (HARRIS *et al.* 1994), limiting MMR (HARRIS *et al.* 1997b), or other (TORKELSON *et al.* 1997; G. J. MCKENZIE, R. S. HARRIS, P. L. LEE and S. M. ROSENBERG, unpublished results).

Independent events underlie mutation of *lac* and chromosomal but not F′-linked genes: Previously, F′-linked as well as chromosomal genes were hypermutated in Lac$^+$ stationary-phase mutants (TORKELSON *et al.*

TABLE 2

***recG* increases mutation specifically in the Lac$^+$ population**

Genotype	Mutants/Lac$^+$ colony Mal$^-$	Xyl$^-$	Mutants/Lac$^-$ colony Mal$^-$	Xyl$^-$
rec$^+$	2/4,080	4/4,080	1/28,301	1/28,301
recG	25/6,427	19/6,427	1/24,036	3/24,036

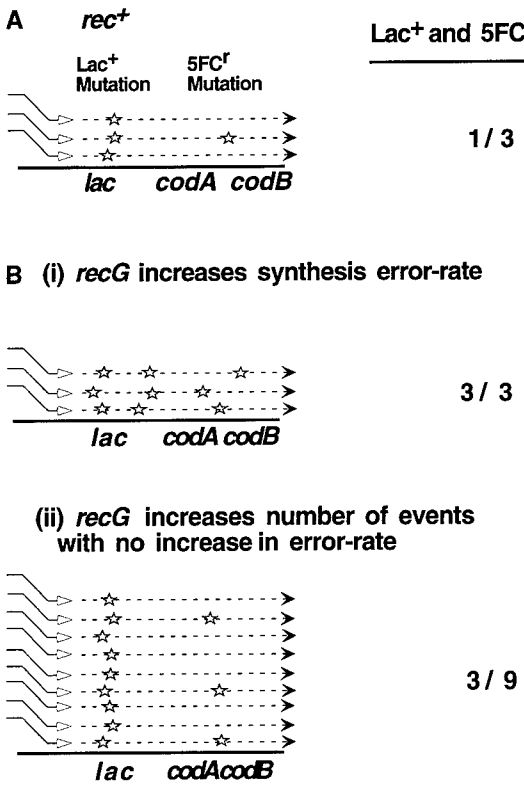

FIGURE 3.—Models for mutation at a locus linked to *lac* on the F′. (A) Proposal that mutation at *lac* and the linked *codAB* locus result from a common DNA replication event. *lac* and *codA codB* are located near each other on the F′. Among all selected mutational events that give rise to Lac$^+$ colonies, some frequency of the time (1/3, as a simple model) an unselected mutation in *codA* or *codB* also occurs, giving rise to a Lac$^+$ FC^r colony. (B) Two models to account for the increase in Lac$^+$ mutations in a *recG* strain. (i) If *recG* increased the error rate of DNA synthesis (for example by disabling DNA polymerase proofreading or postsynthesis mismatch repair), then the number of mutations per base synthesized would increase. As a consequence, the frequency of unselected mutations at *codA*, *codB* would increase among Lac$^+$ colonies. This is not observed (Table 3). (ii) If *recG* increases the number of synthesis events without affecting the error rate, then the ratio of Lac$^+$ 5FC^r (3/9) will not change compared with the *rec*$^+$ situation (1/3). This latter model is supported by the data in Table 3. (- - -) Newly synthesized DNA.

1997). These F′-linked mutations associated with Lac$^+$ could result from recombination-dependent mutation, but might not show increased mutability in *recG* or *recD*, if the recombination event that leads to their formation is the same event responsible for mutation at *lac* (Figure 3). For example, mutations at *lac* and the nearby locus *codAB* might occur via polymerase errors made during the same act of DNA synthesis, from the same recombinational DNA repair event (Figure 3A). If so, their coincident frequency would not be increased by conditions that increase only the number of recombination (and synthesis) events, without increasing the error rate per base synthesized (Figure 3Bii). Both *recD* and *recG* null alleles are expected to increase replicative strand-exchange intermediates (discussed above) and not error rate per base synthesized, as neither has a general mutator phenotype (HARRIS *et al.* 1994, 1996; FOSTER *et al.* 1996).

Unselected mutations at *codAB*, on the F′, were assayed in Lac$^+$ stationary-phase mutants (MATERIALS AND METHODS; TORKELSON *et al.* 1997). In the case of *recG* cells (Table 3), there is a 2-fold increase in the frequency of *codAB* mutation in Lac$^+$ mutants, whereas Lac$^+$ mutation itself was increased 26-fold. These physically linked sites do not show elevated coincident mutation, as the unlinked chromosomal and *lac* sites do (Tables 1 and 2; Figure 2). This implies that the recombination events that generate Lac$^+$ and *codAB*$^-$ mutations are not independent events.

We infer from these data that Lac$^+$ and chromosomal mutations occur during independent events and that both events are stimulated in *recD* and *recG* cells. One implication of these results is that *lac* and the sites mutated in the chromosome do not need to be joined physically (as they would be in an Hfr cell) during chromosomal hypermutation (discussed below).

In *recD*, mutation at *codAB* was increased just over twofold relative to *rec*$^+$, whereas Lac$^+$ mutation increased fourfold (Table 3). Recall that a twofold increase in secondary mutation frequency in *recD* was also seen for chromosomal loci (Table 1 and Figure 2). This suggests that the absence of RecD affects *codAB* and *lac* independently at least for some of the mutation events. Possible bases for these results are discussed below.

DISCUSSION

The results reported here can be summarized as follows:

1. Absence of either RecD or RecG increases concurrent mutation of chromosomal sites in Lac$^+$ stationary-phase mutants (Table 1 and Figure 2).
2. This increase is similar to the increase in Lac$^+$ mutation in *recD* and *recG* strains (Figure 2).
3. The increase in chromosomal mutation frequency is specific to cells that experienced a Lac$^+$ mutation and is not seen in Lac$^-$ starved cells (at least in the case of *recG*; Table 2).

   Because both *recD* and *recG* are predicted to promote strand-exchange recombination intermediates leading to replication (Figure 1, and reviewed above), these data support models in which (some) sites on the bacterial chromosome are accessible to recombination-promoted mutation in stationary phase.
4. The increase in mutability in *recG* is observed at chromosomal sites but not at *codAB* on the F′ (Table 3). This implies that mutations at sites linked to *lac* do not usually occur independently of the Lac$^+$ mutation event. This also implies that *lac* and chromosomal sites are not linked during mutation of chromosomal

TABLE 3

Frequencies of coincident mutation at F′-linked loci *codAB* and *lac* in *recD* and *recG* strains

rec genotype	$Lac^+/10^8$ cells plated (n)[a]	Fold increase (rec/rec^+)[b]	$5FC^r$ mutants/Lac^+ adaptive revertants screened	Fold increase (rec/rec^+)[c]
rec^+	15.6 (5,587)	1.0	18/5,170	1.0
recD	68.72 (11,522)	4.4	86/10,906	2.2
recG	406.3 (8,429)	26	58/8,394	2.0

[a] n, number of Lac^+ mutant colonies scored.
[b] Fold increase in the frequency of Lac^+ reversion.
[c] Fold increase in the frequency of unselected $5FC^r$ mutation in the F′.

sites. We suggest that the same recombination events that lead to Lac^+ mutation also lead to mutation of nearby genes, perhaps in the same DNA recombination-replication event. Evidence that recombination events promote DNA replication directly is reported elsewhere (Motamedi *et al.* 1999 and references reviewed therein; see also Courcelle *et al.* 1997; Kogoma 1997; Liu *et al.* 1999 for further discussion).

Recombination-promoted mutation in the bacterial chromosome: It was suggested that recombination-dependent stationary-phase mutation might be confined to sex plasmids because mutations at *lac* require F′ transfer (Tra) proteins (Foster and Trimarchi 1995a; Galitski and Roth 1995), though not actual transfer (Foster and Trimarchi 1995b; Radicella *et al.* 1995; Rosenberg *et al.* 1995), and because the *lac* operon on the chromosome is cold for recombination-dependent mutation (Foster and Trimarchi 1995a; Radicella *et al.* 1995; Rosche and Foster 1999; M.-J. Lombardo and S. M. Rosenberg, unpublished results). Previous evidence arguing against F′ specificity included, first, hypermutation of chromosomal genes during Lac^+ adaptive mutation (Torkelson *et al.* 1997; Rosche and Foster 1999; this study) and, second, the demonstration of chromosomal hot and cold spots for mutation (Rosenberg 1997; Torkelson *et al.* 1997), which can explain why not all chromosomal sites mutate recombinationally.

The demonstrations that *recG* and *recD* promote coincident chromosomal mutation (Figure 2; Tables 1 and 2) suggest that chromosomal sites are susceptible to recombination-dependent mutation. Note that we cannot test recombination dependence directly because blocking recombination via, *e.g.*, loss of RecA, RecB, or RuvA, B, or C functions abolishes stationary-phase Lac^+ mutation (Harris *et al.* 1994, 1996; Foster *et al.* 1996), and we have scored chromosomal mutations only in cells that are also Lac^+. Thus, although further, direct evidence is required to demonstrate conclusively that recombination-dependent mutation occurs in the *E. coli* chromosome, the current information is most easily explained by such a model.

Independence of Lac^+ and coincident chromosomal mutations: The finding that the coincident mutation frequency of *lac* and chromosomal sites increases in *recD* and *recG* cells (Tables 1 and 2; Figure 2) implies that the mutation frequency at each site is increased by these alleles. These results bear on the possibility that although it occurs in the chromosome, hypermutation during Lac reversion might actually require integration of the F′ into the chromosome. This occurs when Hfr chromosomes form (*e.g.*, Lloyd and Low 1996). We found previously that Lac^+ mutants carrying chromosomal mutations are not enriched for Hfr's (Lombardo *et al.* 1999b). However, we could not rule out the possibility that chromosomal mutations form in short-lived Hfr cells, which subsequently re-form the F′ (Lombardo *et al.* 1999b). The mostly independent stimulation of mutation in chromosomal and *lac* genes by *recD* and *recG* (Tables 1 and 2; Figure 2) does not support such models.

Site-specificity and the role of the F′: We have suggested that the key feature that allows some sites, and not others, to mutate recombinationally is occurrence of DNA DSBs at which RecBCD loads (Harris *et al.* 1994; Rosenberg *et al.* 1995; Rosenberg 1997; Torkelson *et al.* 1997). In this view, Tra proteins activate the F′ by nicking the origin of transfer (Rosenberg *et al.* 1995), and hot and cold sites on the chromosome correspond with sites that are more or less susceptible to DSBs (reviewed by Rosenberg 1997).

Although the results presented here suggest that the F is not needed *in cis* with the DNA that mutates (discussed above), it remains possible that *trans*-acting functions encoded by the F are required for mutation of chromosomal genes. The F encodes several proteins that interact with DNA, including its own single-strand DNA binding protein, a topoisomerase-like double-strand endonuclease, components that modify the bacterial SOS response, and many of the transfer proteins (reviewed by Bagdasarian *et al.* 1992; Frost *et al.* 1994; Yarmolinsky 1995; Firth *et al.* 1996). Whether recombination-dependent stationary-phase mutation and hypermutation of unselected genes can occur in the absence of sex plasmids is not yet known (see *Note added in proof*).

***recD* and coincident mutation in the F′ and chromosome:** *recD* null mutants are hyperrecombinagenic (Chaudhury and Smith 1984; Amundsen *et al.* 1986; Biek and Cohen 1986; Thaler *et al.* 1989), hypermutable in recombination-dependent stationary-phase Lac mutation (Harris *et al.* 1994; Rosenberg *et al.* 1994), and recently have been seen to increase F′ copy number relative to the chromosome (Foster and Rosche 1999). The stationary-phase hypermutation at *lac* in *recD* cells might have resulted from hyperrecombination in *recD* cells (Harris *et al.* 1994) or from more *lac* copies available for mutation in those cells (Foster and Rosche 1999) or from both. The finding that chromosomal gene mutability increases about as much as *lac* does in *recD* cells supports the recombinational idea and does not support the idea of an effect based purely on increased F′ copy number relative to the chromosome.

A perplexing result is that, unlike *recG*, the *recD* effect on chromosomal and F′ sites was similar (Tables 1 and 2). This could indicate a global (stationary-phase specific; Harris *et al.* 1994) twofold mutator activity in *recD* strains. However, another interpretation is possible. Loss of the RecD subunit changes RecBCD enzyme (Amundsen *et al.* 1986; Palas and Kushner 1990) and prevents Chi recognition by the enzyme (Chaudhury and Smith 1984; Thaler *et al.* 1989). Whereas most recombination models include RecBCD-mediated digestion of DNA from a double-strand end up to a Chi site followed by recombination at Chi (*e.g.*, Rosenberg and Hastings 1991; Myers and Stahl 1994; Anderson and Kowalczykowski 1997), in *recD* (exonuclease-defective) cells, the RecBC(D^-) enzyme promotes recombination immediately at the DNA end at which it loads (Thaler *et al.* 1989). This would change the position of strand-invasion events and, in models in which recombination primes replication, would alter the positions of synthesis tracts (Figure 4). Two loci might be synthesized on the same tract in rec^+ cells and on different tracts in *recD* cells (Figure 4), leading to uncoupling of *lac* and *codAB* mutation in *recD* cells.

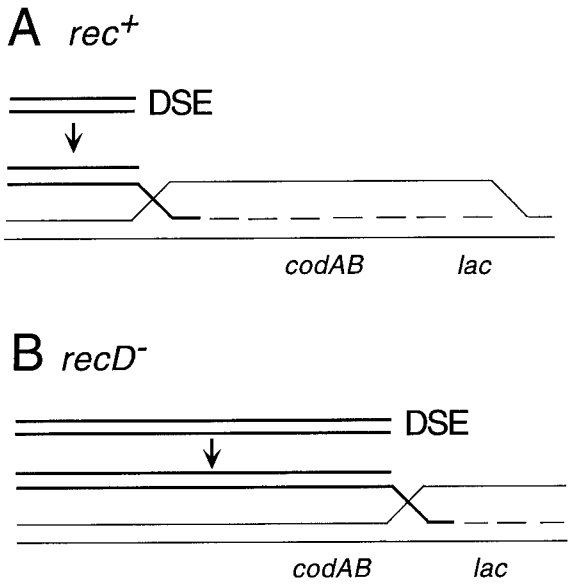

Figure 4.—DSEs and therefore DNA synthesis tracts primed in double-strand break repair would fall in different places in rec^+ and $recD^-$ cells. This is so because the RecBCD enzyme is a double-strand DNA exonuclease whereas the RecBC(D^-) enzyme lacks exonuclease activity, but is recombination-proficient (Chaudhury and Smith 1984; Amundsen *et al.* 1986; Biek and Cohen 1986). Dashed lines represent newly synthesized DNA. See text for discussion.

Implications for the hypermutable subpopulation: *1. Recombination and the hypermutable subpopulation:* Previously, *lac* and an F′-borne gene were observed to show no increase in coincident mutation in *recG* cells, leading to the suggestion that *recG* somehow increases the size of the hypermutable subpopulation, rather than the mutability per subpopulation cell (Foster 1997). Our results for the F′ (Table 3) agree with those reported previously (Foster 1997). However, the data we have obtained on chromosomal site mutability (Table 1 and Figure 2) do not support the idea that *recG* increases subpopulation size, but rather imply that the mutability of subpopulation cells is increased by promoting strand-exchange intermediates. We suggest that at linked sites, the secondary mutation event and the primary Lac^+ mutation event are not independent, such that their coincident mutation frequency does not reflect the mutability per cell.

2. How many mutable subpopulations? Torkelson *et al.* (1997) reported that single (Lac^+), double (Lac^+ plus an additional mutation), and triple mutants (Lac^+ plus two additional mutations) fit a Poisson distribution if a mutation rate of 5×10^{-3} mutations per cell per day occurred in 10^{-4} to 10^{-5} of the cells of the whole population. The ability to fit the data to a Poisson distribution was taken to imply that mutation to Lac^+ and the formation of associated mutations occur at about the same frequency. This is compatible with the hypothesis that Lac^+ and associated mutations occur by the same mechanism and arise from the same subpopulation, but does not exclude the possibility that there are two or more mechanisms affecting different but overlapping subpopulations. A different data set and method of calculation led to the conclusion that only 10% of the Lac^+ mutations result from the hypermutating subpopulation that gives rise to the secondary mutations (Rosche and Foster 1999). However, the data on associated mutations were few, such that with 95% confidence limits applied to them, as many as 98% of the Lac^+ mutants could have arisen from the hypermutable subpopulation.

Nevertheless, the general concept of different but overlapping subpopulations may be applied to the results presented here. The *recD* and *recG* mutations might increase the size of the subpopulation undergoing mutation to Lac^+ such that the subpopulation now includes

a higher proportion of those cells of the subpopulation that gives rise to associated mutations. This would have the effect of increasing the frequency of associated mutations among the Lac^+ mutants without increasing the mutation rate in the hypermutating subpopulation. Invoking two populations and two mechanisms is a more complicated and thus less attractive model.

3. Subpopulation size and mutation rate: The proposed mutation rate of 5×10^{-3} mutations per cell per day of TORKELSON *et al.* (1997) may seem lethally high, and yet no net cell death is observed (CAIRNS and FOSTER 1991, and many subsequent references). It should be noted, however, that first, even massive death of a subpopulation of 10^{-5} of the cells would be unnoticeable when measuring cell viability and, second, because only some (hot) sites are mutable (see discussion of hot and cold sites above in ROSENBERG 1997), many essential genes may be spared, so death might not occur (supported by data of FOSTER 1997).

Significance: The findings reported here suggest that recombination-dependent stationary-phase mutation is a mechanism of genetic change under stress that can alter at least some of the cell's primary genetic reserve, the chromosomal genes. This inference will hold whether or not components on the F′ are found to be required for the chromosomal hypermutation. Sex plasmids are natural genetic elements and if they provide such conditional mutability to their hosts, this could be an advantageous, selected feature for their host cells.

Several aspects of recombination-dependent stationary-phase mutation may also be general to other organisms and circumstances. Mutation promoted by DSB-repair recombination in yeast has been demonstrated (STRATHERN *et al.* 1995; HOLBECK and STRATHERN 1997), as has recombinational involvement in mutation in vertebrates including mammals (reviewed by MAIZELS 1995; HARRIS *et al.* 1999b). Findings suggestive of this association abound in many organisms (DEMEREC 1962, 1963; MAGNI and VON BORSTEL 1962; PASZEWSKI and SURZYCKI 1964; ESPOSITO and BRUSCHI 1993). Additionally, the MMR system, which becomes limiting during stationary-phase mutation (HARRIS *et al.* 1997b, 1999a), is conserved in eubacteria and eukaryotes. Its loss of function is also a powerful force of genetic change in other organisms (reviewed by RADMAN *et al.* 1995; KOLODNER 1996; MODRICH and LAHUE 1996), and its transient diminution would be potentially more important in multicellular organisms that suffer more drastic consequences from mutagenesis of component cells. The mechanism of action, control, and scope of this stationary-phase mutation mechanism in *E. coli* will illuminate a path toward understanding conditional mutagenesis, programmed or accidental, in all of these systems.

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Note addeed in proof: Recent work of GODOY *et al.* (2000) both confirms previous findings of chromosomal hypermutation during Lac^+ adaptive mutation (TORKELSON *et al.* 1997; ROSCHE and FOSTER 1999) and indicates that there is indeed an F′-supplied function that promotes stationary-phase mutation. As discussed above, the results presented here imply that any F′-related function would act *in trans* in mutation, not via Hfr formation (above, and LOMBARDO *et al.* 1999b).

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The SOS response regulates adaptive mutation

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Upon starvation some *Escherichia coli* cells undergo a transient, genome-wide hypermutation (called adaptive mutation) that is recombination-dependent and appears to be a response to a stressful environment. Adaptive mutation may reflect an inducible mechanism that generates genetic variability in times of stress. Previously, however, the regulatory components and signal transduction pathways controlling adaptive mutation were unknown. Here we show that adaptive mutation is regulated by the SOS response, a complex, graded response to DNA damage that includes induction of gene products blocking cell division and promoting mutation, recombination, and DNA repair. We find that SOS-induced levels of proteins other than RecA are needed for adaptive mutation. We report a requirement of RecF for efficient adaptive mutation and provide evidence that the role of RecF in mutation is to allow SOS induction. We also report the discovery of an SOS-controlled inhibitor of adaptive mutation, PsiB. These results indicate that adaptive mutation is a tightly regulated response, controlled both positively and negatively by the SOS system.

DNA repair | *Escherichia coli* | signal transduction | RecF | RecA

The bacterial SOS response, studied extensively in *Escherichia coli*, is a global response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced (1). SOS is the prototypic cell cycle check-point control and DNA repair system, and because of this, a detailed picture of the signal transduction pathway that regulates this response is understood. A central part of the SOS response is the de-repression of more than 20 genes under the direct and indirect transcriptional control of the LexA repressor. The LexA regulon includes recombination and repair genes *recA*, *recN*, and *ruvAB*, nucleotide excision repair genes *uvrAB* and *uvrD*, the error-prone DNA polymerase (pol) genes *dinB* (encoding pol IV) (2) and *umuDC* (encoding pol V) (3), and DNA polymerase II (4, 5) in addition to many functions not yet understood. In the absence of a functional SOS response, cells are sensitive to DNA damaging agents.

The signal transduction pathway leading to an SOS response (reviewed by ref. 6) ensues when RecA protein binds to single-stranded DNA (ssDNA), which can be created by processing of DNA damage, stalled replication, and perhaps by other means (7–9). The ssDNA acts as a signal that activates an otherwise dormant co-protease activity of RecA, which allows activated RecA (called RecA*) to facilitate the proteolytic self-cleavage of the LexA repressor, thus inducing the LexA regulon (10). Activated RecA also facilitates the cleavage of phage repressors used to maintain the quiescent, lysogenic state, and UmuD, creating UmuD′, the subunit of UmuD′C (pol V) that allows activity in trans-lesion error-prone DNA synthesis (6).

An intriguing feature of the SOS response is inducible mutation (11, 12). LexA-repressed pol V participates in most UV mutagenesis, by inserting bases across from pyrimidine dimers (3). Pol IV is required for an indirect mutation phenomenon in which undamaged phage λ DNA is mutated when added to UV-irradiated (SOS-induced) cells (13). There may be other mutagenic mechanisms induced by the SOS response.

Adaptive mutation (also called stationary-phase mutation) is a collection of phenomena in which mutations form in stressed or starving, nongrowing, or slowly growing cells, and at least some of these mutations allow growth (reviewed by refs. 14–19). It is a model for mutational escape of growth-control, such as in oncogenesis, tumor progression, and resistance to chemotherapeutic drugs (16, 20–22), and also, like SOS mutagenesis, implies that evolution can be hastened when the need arises (23).

Adaptive mutation has been studied most extensively using an assay for reversion of a *lac* +1 frameshift allele on an F′ sex plasmid in *E. coli* starved on lactose medium (24). The adaptive mutations are unlike Lac$^+$ mutations in growing cells in that they form during (not before) exposure to selective conditions (25), and occur via a unique molecular mechanism (reviewed by refs. 18 and 19) that requires homologous recombination proteins RecA, RecBC, and RuvABC (22, 26, 27). The adaptive mutations occur in a hypermutable subpopulation of the starved cells (28–30) during a transient period of limiting mismatch-repair activity (31) and possess a unique sequence spectrum of −1 deletions in mononucleotide repeats (32, 33) identical to that of mismatch repair defective cells (34).

As reviewed above, the cells undergoing adaptive mutation are transiently differentiated and mutable. However, the mechanism(s) by which the environment induces this differentiation, the signals from the environment, and the signal transduction pathway(s) provoking adaptive mutation are unknown. We have examined the role of the SOS response in adaptive mutation and report both positive and negative control of adaptive mutation in the Lac system by the LexA repressor. First, we report that SOS induction of the LexA regulon is required for efficient adaptive mutation. Simple overproduction of RecA, a recombination protein controlled by LexA, does not substitute. Second, we provide evidence that RecF protein is required for efficient mutation in its SOS-inducing capacity. This implies that the DNA signal provoking SOS during adaptive mutation is not a DNA double-strand break (DSB) as postulated previously (e.g., ref. 18), and implies that there are ssDNA intermediates in mutation other than at DSBs. Third, we find evidence of an SOS-controlled repressor of adaptive mutation, PsiB, a protein known to inhibit RecA* activity. The adaptive mutation response appears to occur within a narrow window in the continuum of levels of SOS induction. These results (*i*) indicate that adaptive mutation is a tightly regulated response, (*ii*) identify part of the signal transduction pathway that controls it, and (*iii*) illuminate possible DNA intermediates in that signal transduction pathway.

Abbreviations: ssDNA, single-stranded DNA; pol, polymerase; DSB, double-strand break.

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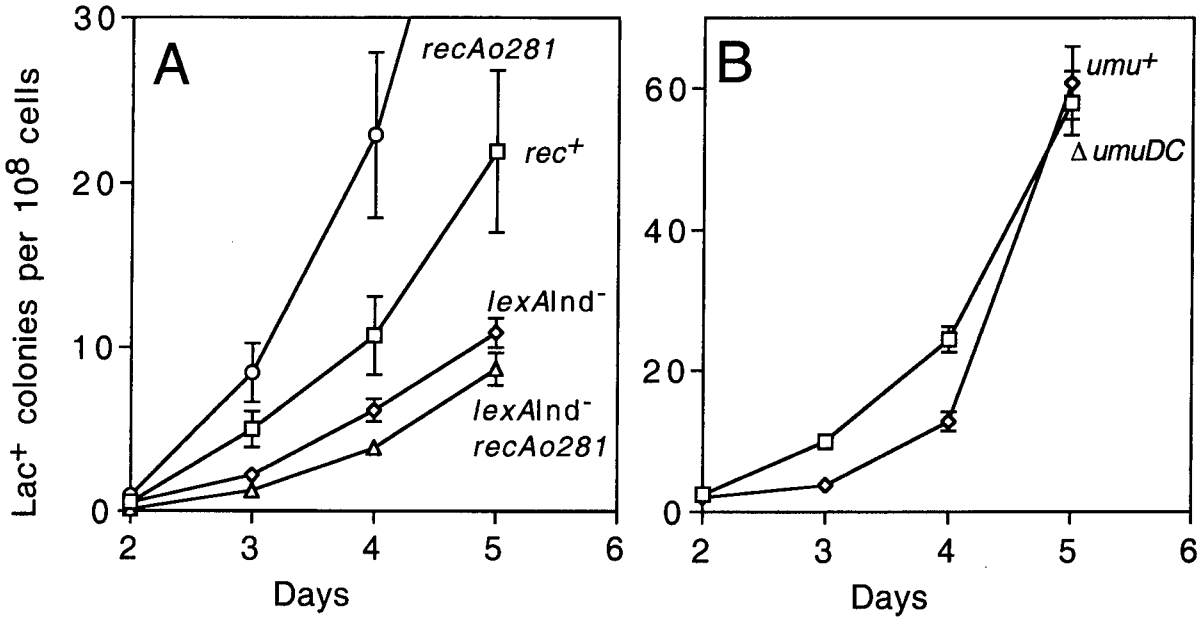


Fig. 1. (*A*) Induction of a LexA-regulated gene(s) other than or in addition to *recA* is required for efficient Lac$^+$ adaptive mutation. ○, *recAo281*; □, *rec*$^+$; ◇, *lexA3(Ind*$^-$*)*; △, *lexA3*(Ind$^-$) *recAo281*. (*B*) *umuDC* is not required for adaptive mutation. □, *Δ(umuDC)595::cat*; ◇, *umu*$^+$. In this and all figures, all strains shown together were tested in parallel, and the means ± SEM (error bars) of several independent cultures tested in parallel are displayed.

Materials and Methods

All new *E. coli* strains were constructed using standard P1 transduction techniques (35). The mutant alleles used were *recAo281* (36), *lexA3*(Ind$^-$) (37), *lexA51*(Def) (38), *sulA211* (*E. coli* Genetic Stock Center, New Haven, CT), *recF332*::Tn*3* (39), *dinI::kan* (40), *psiB::cat* (A. Bailone, Orsay, France), and *Δ(umuDC)595::cat* (41). Strains used in the mutation assay are derived from FC40 (24), which carries a deletion of the chromosomal *lac-pro* region and an F′ carrying *pro*$^+$ and a *lacI33ΩlacZ* fusion with a +1 frameshift mutation such that the cells are phenotypically Lac$^-$. Mutation assays were as described previously (27), including that cell viability measurements for all experiments reported showed no net growth or death of the frameshift-bearing cells. Some variability is seen in absolute values from experiment to experiment, but relative values between strains remained the same within a minimum of three repeats. Single representative experiments are shown (see Figs. 1–3) and the consistency of results across multiple repeats summarized (see Fig. 4).

Results

Induction of a LexA Controlled Gene(s) Other Than or in Addition to RecA Is Required for Adaptive Mutation. The *lexA3*(Ind$^-$) allele encodes a noncleavable mutant LexA protein (42, 43) containing a substitution of Gly-84 to Asp (44, 45). In *lexA3*(Ind$^-$) cells, the LexA regulon is repressed and cannot be induced. In a strain carrying *lexA3*(Ind$^-$), adaptive mutation is decreased 3- to 4-fold (Fig. 1*A*), as seen previously (24). This result indicates a requirement for induced levels of a LexA-repressed gene(s) for efficient adaptive mutation. The LexA-repressed gene(s) could be required absolutely for adaptive mutation if the basal level of expression in uninduced cells is sufficient for some adaptive mutation to occur.

recA is repressed by LexA, and is induced >10-fold during the SOS response (1). RecA is essential for adaptive mutation (22), making it a reasonable candidate for being required at induced levels. To test this hypothesis, we used a *recA* operator-constitutive allele, *recAo281*, that produces induced levels of RecA constitutively (36). In *lexA3*(Ind$^-$) *recAo281* cells, RecA is produced at levels similar to those during SOS induction (36). This allele does not restore the level of adaptive mutation in *lexA3*(Ind$^-$) cells to the level of *lexA*$^+$ cells (Figs. 1*A* and 4*A*), in contrast with data reported previously (24). The strain used by those authors was shown subsequently not to carry *lexA3*(Ind$^-$) (26, 46). This failure to restore mutation with a

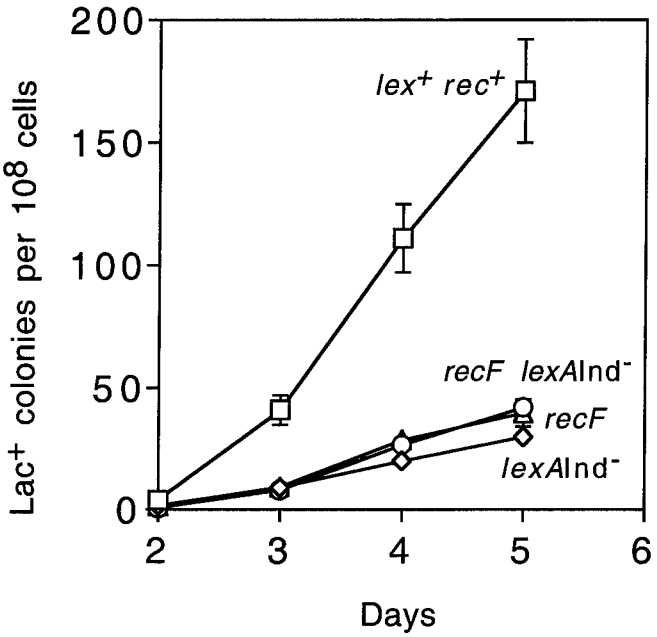
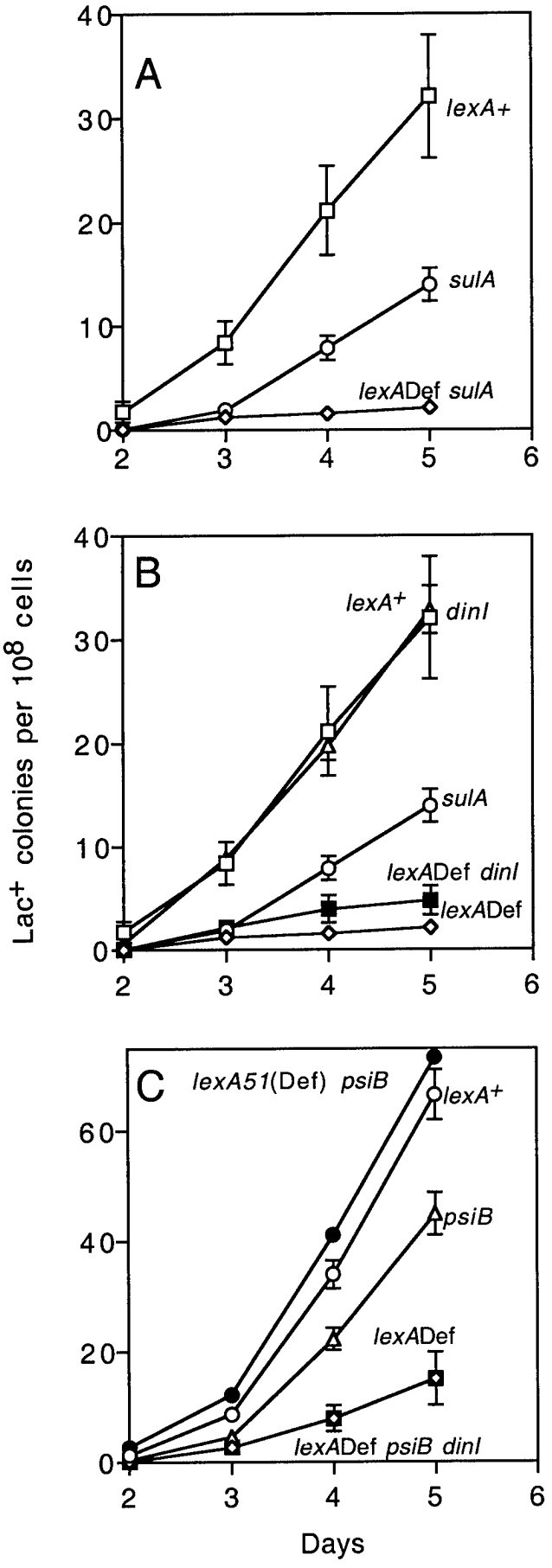


Fig. 2. RecF$^+$ promotes adaptive mutation and acts via the same pathway as LexA. □, *rec*$^+$ *lexA*$^+$; △, *recF332*::Tn*3*; ○, *recF332*::Tn*3 lexA3(Ind*$^-$*)*; ◇, *lexA3(Ind*$^-$*)*.



constitutively expressing *recA* allele indicates that *recA* is either not the LexA-repressed gene, or not the only LexA-repressed gene, required at induced levels for efficient adaptive mutation.

The LexA-repressed function required at induced levels is not the mutagenic UmuDC complex (Fig. 1*B*). This agrees with work showing that a *recA* allele unable to cleave UmuD to the mutagenically active form, UmuD′, does not affect the level of adaptive mutation (24).

RecF Is Required for Efficient Adaptive Mutation. RecF protein plays a poorly defined role(s) in recombination *in vivo* (47, 48). RecF is also required for SOS induction by some DNA damaging agents that produce single-stranded lesions (and not those that produce DSBs) (49). In a *recF* mutant, adaptive mutation is decreased 3- to 5-fold (Fig. 2 and 4*B*). To test whether RecF promotes adaptive mutation via its recombination capacity or via allowing SOS induction, a *recF lexA3*(Ind$^-$) strain was examined. *recF* is epistatic with *lexA3*(Ind$^-$) (Fig. 2), suggesting that the role of RecF in mutation is to allow SOS induction and not via recombination (alternatives discussed below).

A LexA-Controlled Inhibitor of Adaptive Mutation. Because induction of some protein(s) is required, we tested whether constitutive de-repression of the LexA-repressed genes promotes adaptive mutation. Cells lacking LexA must also carry a mutation in the *sulA* gene to be viable because SulA is a LexA-repressed protein that inhibits cell division (50). A *sulA* mutation by itself decreases adaptive mutation slightly (Figs. 3*A* and 4*C*). This could be because more cell division occurs during SOS in the absence of SulA, such that sister chromosomes have more opportunity to segregate and thus less opportunity to recombine. Sister chromosomes are a possible source of the homologous DNA used in the recombination required for adaptive mutation in this system (22). In contrast to the simplest prediction, the *lexA51*(Def) *sulA* cells show greatly decreased adaptive mutation (Fig. 3*A*). This finding indicates that constitutive de-repression of some LexA-repressed gene(s) inhibits adaptive mutation.

We tested two candidates for the LexA-repressed inhibitor(s) of adaptive mutation. DinI is a LexA-repressed protein that inhibits recombination and SOS induction by binding and altering RecA (40). Its proposed function is to help return cells to normal after an SOS response. We find that loss of *dinI* in a *lexA51*(Def) cell has little effect (Figs. 3*B* and 4*C*), indicating that DinI is not an important LexA-repressed inhibitor of adaptive mutation. However, a different anti-SOS protein encoded by the F plasmid, PsiB (51), appears to be the LexA-repressed inhibitor. In the absence of LexA, the loss of PsiB restores adaptive mutation to normal (Figs. 3*C* and 4*C*). PsiB also interacts with RecA to decrease RecA* activity (51). In addition, loss of PsiB in *lexA*$^+$ cells diminishes adaptive mutation. This finding implies that the extent of RecA* activity is crucial to adaptive mutation, indicating a tight regulatory control over adaptive mutation, as does the following result. When *psiB* and *dinI* are both removed in a *lexA51*(Def) strain, adaptive mutation is diminished greatly relative to *psiB lexA51*(Def) (Figs.

Fig. 3. LexA-repressed inhibitor(s) of Lac$^+$ adaptive mutation. (*A*) Complete de-repression of the LexA regulon inhibits mutation. The *lexA* defective strain carries *lexA51*(Def) *sulA211* (◇), *lexA*$^+$ (□), and *sulA211*(○). The *sulA* mutation, required for viability of *lexA51*(Def) strains, also depresses mutation modestly (discussed in text). (*B*) The LexA-controlled inhibitor of adaptive mutation is not DinI. Both *lexA51*(Def) strains also carry *sulA211*. □, *dinI*$^+$ *lexA*$^+$; △, *dinI*::*kan lexA*$^+$; ○, *sulA211*; ■, *lexA51*(Def) *sulA211 dinI*::*kan*; ◇, *lexA51*(Def) *sulA211*. (*C*) PsiB inhibits adaptive mutation in LexA de-repressed cells. All strains shown carry *sulA211*. Additional alleles carried are as follows: ○, *lexA*$^+$; ●, *lexA51*(Def) *psiB*::*cat*; △, *psiB*::*cat*; ◇, *lexA51*(Def); ■, *lexA51*(Def) *psiB*::*cat dinI*::*kan*. Results are discussed in the text.

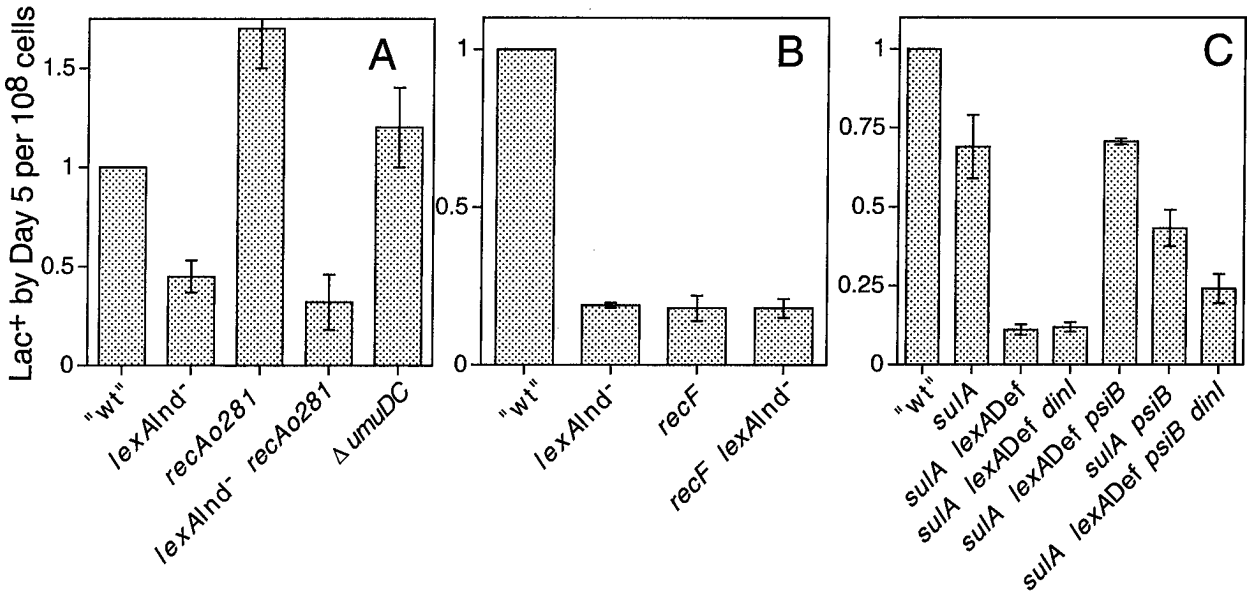


Fig. 4. Comparison of various SOS altered mutants with "wild-type" (wt) in multiple experiments. The fold-differences in accumulation of Lac^+ mutant colonies by day 5 is displayed between of each of the mutants listed and the nonmutant parental strain (wt; set equal to 1) assayed in parallel over multiple experiments. The numbers (n) of experiments averaged (error bars equal 1 SEM) are as follows: wt, $n = 4$; *lexA3*(Ind^-), $n = 4$; *recAo281*, $n = 4$; *lexA3*(Ind^-) *recAo281*, $n = 4$, *ΔumuDC*, $n = 3$ (*A*); $n = 3$ for all genotypes displayed (*B*); and $n = 3$ for all genotypes displayed except "wt" and *sulA lexAdef* for which $n = 4$ (*C*). "wt" is isogenic with all of the mutants used, and carries wild-type alleles of all *rec*, *lex*, *sul*, *din*, and *psi* genes and Δ(*pro-lac*) Rif *thi* F′ *pro*$^+$*lacIq lacI33*Ω*lacZ*.

3*C* and 4*C*). This result suggests that *psiB* is not a direct inhibitor of adaptive mutation, but modulates it by modulating the SOS response, and that adaptive mutation is very sensitive to the extent and/or duration of the SOS response, and to levels of RecA* activity. Because RecA* activity is implicated as being important even when the LexA regulon is fully derepressed [in *lexA51*(Def) cells], these results might imply that a target of the RecA* co-protease activity in addition to LexA is important in adaptive mutation (this and alternatives are discussed below).

To summarize: (*i*) PsiB appears to inhibit adaptive mutation when the LexA regulon is constitutively de-repressed in a *lexA*Def mutant; and (*ii*) two proteins that modulate RecA* activity, DinI and PsiB, affect adaptive mutation positively and negatively. These data suggest that RecA* activity is critical in adaptive mutation, that if RecA* activity is either too high or too low, mutation is decreased. These results indicate a tight control over adaptive mutation by factors modulating the SOS response, and provide evidence of SOS regulation of adaptive mutation independent of particular LexA alleles.

Discussion

The results reported indicate that adaptive mutation in the Lac system in *E. coli* is regulated by the SOS system. This identifies SOS as a signal transduction pathway controlling the transient, differentiated condition (52) of adaptive mutation, and likewise identifies adaptive mutation as a new form of SOS mutagenesis.

The LexA Regulon and Adaptive Mutation. We have shown that efficient Lac adaptive mutation requires SOS-induced levels of a LexA-repressed function(s) other than or in addition to RecA (Fig. 1). As discussed above, no real conflict exists between previously reported data (24) and ours.

Two Roles for RecA. RecA is both a signal sensor/transducer molecule for the SOS response and an important recombination protein (53). Because recombination proteins RecBCD, RuvAB, and RuvC are also required for adaptive mutation (22, 26, 27), and RuvAB and RuvC do not affect SOS induction (1), a recombinational role for RecA in adaptive mutation has been clear. The current results indicate that the SOS activation function of RecA is also required for efficient adaptive mutation. These data allow one to understand the previously perplexing finding that a special *recA* mutation conferring recombination-proficiency and SOS deficiency reduces adaptive mutation in this system (24).

DNA Intermediates in Signal Transduction. Efficient SOS induction requires either RecBC or RecF, depending on whether the DNA intermediate that triggers the SOS response is a double-strand end (RecBCD) or ssDNA not at a double-strand end (RecF) (49). RecF is partially required for adaptive mutation (Fig. 2), and the data suggest that this requirement reflects a requirement for RecF in SOS induction during adaptive mutation: (*i*) loss of RecF decreases adaptive mutation to the same (partial) extent as the LexA-uncleavable mutation (Figs. 2 and 4*B*); and (*ii*) RecF deficiency does not reduce mutation further in a strain that is already LexA uncleavable (Figs. 2 and 4*B*), as expected if the sole function of RecF in mutation is to promote LexA cleavage. The converse possibility, that LexA induction is required to produce RecF, is unlikely because RecF is not thought to be LexA regulated (1). Although not ruled out by our data, schemes in which LexA is imagined to function in a RecF-specific recombination route are more complicated, and so are not favored.

The indication that the RecF function in adaptive mutation is to promote the SOS response implies that the ssDNA signal inducing SOS during adaptive mutation is not at a double-strand end (DSE). This is surprising considering that adaptive mutation in this system absolutely requires RecBC (22), an enzyme that operates only at DNA DSEs and breaks (DSBs), and which catalyzes recombinational DSB-repair in *E. coli* by generation of ssDNA at DSEs (54, 55). One possible explanation is that the timing of SOS induction in adaptive mutation necessarily precedes DSB formation. Another is that perhaps, although DSBs form, single-strand lesions are more abundant during adaptive mutation, and so are more important SOS-inducing signals. Whichever is the case, these results allow us to infer a new DNA

intermediate in adaptive mutation: ssDNA other than single-strands exposed at double-strand ends. DSEs (22) and Holliday junctions (26, 27) are the only other DNA intermediates implicated in adaptive mutation, to date.

The ssDNA-inducing SOS during adaptive mutation could be exposed at nicked DNA at the F′ origin of transfer, stalled replication forks or chemically damaged DNA. If nicks at the F′ transfer origin are the signal, this could explain why transfer (Tra) proteins (but not actual transfer) are required for efficient adaptive mutation (56, 57), despite evidence that the F′ need not be covalently linked with the DNA undergoing mutation (28, 58, 59). A *trans* role for the F′ (also suggested by ref. 30), such as inducing *trans*-acting SOS proteins, seems sensible. Further work will be required to determine when, where, and how the ssDNA signal is generated.

Positive and Negative Control. It was surprising to find that in addition to LexA-controlled factor(s) that promote adaptive mutation, there is a LexA-repressed inhibitor, PsiB (Fig. 3). PsiB is a RecA co-protease inhibitor encoded by the F plasmid (51) and may be repressed by LexA (implied by our data, see Fig. 3*C*). The chromosomally encoded DinI protein also blocks RecA co-protease activity and recombination (40). Both of these proteins may promote a speedy return to the non-SOS state after the DNA damage that induced the response has been repaired. The *dinI* deletion had no effect on mutation in either *lexA51*(Def) or *lexA*$^+$ cells, but decreased mutation in the absence of PsiB (Figs. 3*C* and 4*C*). This finding may imply that DinI competes poorly for RecA binding in the presence of PsiB. This apparently perplexing result suggests that levels of RecA* are crucial to successful adaptive mutation. For example, adaptive mutation might be regulated temporally by the SOS response, with both early entry (in LexA-defective cells) and early exit (PsiB$^+$) or late exit (PsiB$^-$ DinI$^-$) from the SOS response being inhibitory to adaptive mutation. Alternatively, cells lacking both PsiB and DinI may simply not survive the SOS induction and hypermutation to form (Lac$^+$) colonies, as follows.

SOS and Hypermutability Are Differentiated States. Recombination-dependent adaptive mutation occurs in a hypermutable subpopulation of the stressed cells (10^{-4} to 10^{-5}) (28, 59). We suggest that SOS induction may be the event that differentiates subpopulation cells from the main population. Although no net cell death was observed during the experiments with the *dinI psiB* strain (see *Materials and Methods*), death of only the subpopulation would have been undetectable.

The discovery that the LexA regulon includes both repressor(s) and promoter(s) of adaptive mutation implies that adaptive mutation is a tightly regulated process. SOS is the first signal transduction pathway found to control adaptive mutation in this system.

Candidate Genes and Molecular Mechanism. The LexA-repressed gene(s) needed at induced levels for efficient Lac-adaptive mutation have not been identified. However, some plausible candidates are suggested by our current picture of the molecular mechanism of adaptive mutation in this system (17–19). The mutations are suggested to result from DNA polymerase errors that occur during the DNA replication (22) now known to be associated with some recombinational double-strand break-repair in *E. coli* (60). The source of the DSBs in the starving cells is not yet known. DSBs may result from stalled replication (22, 61, 62), processing of single-stranded nicks at the F′ transfer origin (63, 64), endonucleases, or chemical damage, or other (e.g., ref. 65). Mismatch repair activity is diminished transiently (28, 34, 52) in the stressed, mutating cells due to a transient limitation of MutL (31, 66). This allows the errors to be fixed as mutations. DNA pol III is implicated in the replication (60, 67, 68). Finally, the mutational process occurs in a small subpopulation of the stressed cells, in which hypermutation occurs at hotspots (not uniformly; ref. 19) throughout the bacterial genome (28–30, 59).

There are several candidate LexA-regulated genes (apart from RecA) whose induction might promote this adaptive mutation mechanism. (*i*) RuvAB recombination proteins (1, 55) are required absolutely for mutation in this system, presumably for the recombination that promotes DNA replication (26, 27). These are expressed constitutively, and may not need to be induced for full recombination (see ref. 69). (*ii*) We found that loss of the SulA cell division inhibitor protein (50, 70) reduces adaptive mutation slightly. Perhaps inhibition of cell division increases the chance of recombination between sister DNA molecules, or lack of division control results in death of some of the subpopulation, which would not be measurable in cell viability determinations. (*iii*) An attractive possibility is the LexA-repressed mutagenic DNA polymerase pol IV, encoded by *dinB* (2, 71). LexA represses three DNA polymerases. Of them, pol II (high accuracy polymerase) inhibits Lac adaptive mutation (46, 67), as if it competes with the mutagenic polymerase that makes the mutations. Pol V (UmuD′C, an error prone polymerase) has no effect (Fig. 1*B*; ref. 24), and pol IV is currently being examined. Pol IV is required for phage λ untargeted mutagenesis (13), and when overexpressed, increases spontaneous mutations (especially −1 frameshifts) up to 800-fold (72). Although DNA pol III is implicated in adaptive mutation (67, 68), the data do not rule out the possibility that another polymerase makes the mutations, or that adaptive mutations are made by both pol III and pol IV (73).

Generality. This report describes the second example of SOS mutagenesis in starving cells independent of UmuDC, both of them dependent on RecA and RecBC. In the first example, aging colonies induce SOS and mutation (74, 75). That SOS response requires cAMP, a signal molecule produced during starvation, and RecB. This is similar to recombination-dependent adaptive mutation (studied here), but the two mutation routes have some different genetic requirements (reviewed by ref. 18) and may represent closely related SOS mutagenesis mechanisms promoted by starvation. UmuDC-dependent SOS transversion mutagenesis in starving cells has also been described (76, 77). Other stationary-phase stress- or starvation-induced mutagenesis mechanisms exist in prokaryotes and eukaryotes (reviewed by refs. 17 and 18), and there are many examples in the literature of recombination-associated mutation in eukaryotes (reviewed in refs. 17, 18, 52, and 78). Components of the regulatory mechanisms of these processes have been described only for transcription-associated mutation, which involves the stringent response (amino acid starvation) (79, 80), SOS-mutagenesis in aging colonies (74, 75) and starving cells (76, 77), *phoPQ* involvement in *ebgR* mutation (81), and this report. Understanding the regulation of all of the different adaptive or stationary-phase mutation mechanisms will illuminate when, how, and whether cells adjust their mutation rates and mechanisms, thereby inducing heritable changes, and presumably increasing their options for survival.

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